ADRENAL → GONAD INTERACTIONS IN THE MALE RAT: STUDIES ON THE INFLUENCE OF THE ADRENAL GLAND ON TESTICULAR STEROIDOGENESIS

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For William, Richard and Sam.

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ABSTRACT

The idea that adrenal secretions can influence the reproductive system has been the subject of a great deal of research effort that spans the last three decades. These basic experiments employed indices of fertility and sexual function as relatively crude end-points for investigation whilst others concentrated on the effects of stress to implicate a role for the adrenal gland in reproduction. However, the introduction of specific radioimmunoassays for steroids in blood and the development of isolated cell technology have permitted new and powerful tools with which to investigate this intriguing problem.

The aim of this thesis is to investigate the mechanisms by which the adrenal gland may influence testicular steroidogenesis in the male rat. The present study has employed and adapted specific radioimmunoassays for progesterone, corticosterone and testosterone for use in rat plasma in addition these assays as well as those for 17α-hydroxyprogesterone and androstenedione, for use in *in vitro* isolated cell studies. Furthermore, the development of a reproducible isolated-cell superfusion technique has allowed this study to focus on possible direct interactions between the steroid-secreting cells of the adrenal and the testis.

The administration of pharmacological doses of glucocorticoid to male rats *in vivo* not only suppresses adrenal steroidogenesis but also inhibits testicular steroidogenesis by inhibiting the anterior pituitary secretion of LH. In contrast, stimulation of the adrenal gland by the administration of ACTH to these pharmacologically-suppressed rats stimulates the adrenal secretion of progesterone and testicular steroidogenesis. The mechanism by which ACTH increases testicular steroidogenesis is dependent on the presence of the adrenal gland and its secretion of progesterone. This thesis draws upon existing evidence of the common elements in adrenal and testicular steroidogenesis and demonstrates that common steroid precursors secreted by the adrenal can be utilised by the testis in the synthesis and secretion of testosterone.

Furthermore, the *in vitro* juxtaposition of ACTH-stimulated isolated adrenal cells with isolated testicular cells results in increased secretion of testosterone and this effect is dependent upon the adrenal secretion of progesterone.

The overwhelming conclusion from these data is that the adrenal gland has an important role in generating external signals that modulate the hypothalamic-pituitary-gonadal axis in male rats. The adrenal secretion of glucocorticoid acts as a negative signal to testicular steroidogenesis whereas progesterone acts as a positive signal. The adrenal secretion of progesterone and its conversion to testosterone by steroidogenic enzymes in the cytoplasm of the Leydig cell may provide an alternative pathway for testosterone biosynthesis and may account for the increased plasma testosterone levels during the acute phase of stress and mating.

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Chapter 1

INTRODUCTION

1.1: The aim of the thesis

The experiments described in this thesis are designed to demonstrate that the adrenocortical secretion of steroid hormones can, either directly or indirectly, modify the testicular secretion of testosterone in the rat.

The idea that adrenal secretions can affect the reproductive system has been the subject of a great deal of research effort since the 1950s (for review see Ramaley, 1974). Evidence that the adrenal gland can influence gonadal relationships, in both sexes, in terms of the development of puberty and sexual maturation (Ramaley, 1974; Meijs-Roelofs, 1978) and gonadal function in general (Andrews, 1977; Kime, 1981) has been extensively reviewed. The experimental approaches employed for the study of adrenal → gonadal interactions have been various. However, this concept has not been widely accepted, primarily because much of the evidence has been indirect. Many of these basic experiments employed indices of fertility and sexual function as relatively crude end-points for investigation, namely the induction of puberty, ovulation, conception, fetal wastage and lactation. Other reviews have concentrated on the effects of stress upon the reproductive system to implicate a role for the adrenal gland in reproduction (Andrews, 1977). From the results of these experiments it is reasonable to argue that the adrenal gland may have an effect upon gonadal function. However, the mechanism(s) by which the adrenal gland may influence gonadal function has not been determined. There is clearly scope for further investigation and the purpose of

this thesis is to investigate the possible influence of the adrenal gland upon testicular steroidogenesis in the rat.

This chapter will review the pathways and regulation of adrenocortical and testicular steroidogenesis, the common elements in these pathways in adrenal and gonadal tissues, and previous studies to date that have implicated a role for adrenocortical steroids in the regulation of testicular steroidogenesis in the rat.

'There can be few more reliable methods of alienating the interest of a scientific or medical audience than by presenting the formulae of half-a-dozen steroid compounds. Perhaps it is the asymmetry of the steroid skeleton or the fact that closely related structures may represent compounds with greatly different chemical, biochemical and physical properties that induces the sense of frustration. This response is quite commonplace, but those who follow the few simple rules and conventions will discover in the steroid group the most orderly and rational arrangement of compounds of any group in the biochemical field; they will have joined the select group for whom the cyclopentanoperhydro-phenanthrene skeleton has no terrors'.

(Kellie, 1984)

Fortunately, steroidogenesis in the rat adrenal cortex and testis is relatively simple, when compared with other mammalian counterparts (for review see Peron, 1968; Chubb, 1979b).

1.2: The Adrenal Cortex

1.2.1: Historical perspectives

The adrenal glands were noted for the first time in 1563 when Bartholomaeus Eustachius named them as 'Glandulae Renibus incumbentes'. However, it was not until 1714 that Eustachius' drawings of human anatomy were first published by Lancisius who named the adrenal glands 'Renes-succenturiati ab Eustachio primum detecti'. Although other names occurred over the ensuing years it was Jean Riolan the younger who introduced the familiar term 'suprarenal capsule' in 1629. The division between the medulla and cortex was recognised by Cuvier in 1805 but the actual terms 'medulla' and 'cortex' were not introduced until 1854 by Hushke, Kölliker and Gray. The use of the term 'adrenal' by Huxley and Martin finally emerged in 1875 (source, Chester Jones, 1976).

1.2.2: Adrenocortical structure and function

In 1858 Harley first described the divisions of the human adrenal cortex into three distinct zones - glomerulosa, fasciculata and reticularis. Over the years information from animal and human studies has established that cells of the zona glomerulosa secrete aldosterone (Ayres, 1956) but studies suggesting a clear separation of structure and function in the zonas fasciculata and reticularis

are somewhat lacking (Bell, 1978; Bell 1980; Maroulis, 1980). Any differences that may exist in the secretion of glucocorticoid and androgen from human adrenal zona fasciculata and reticularis cells appear to be quantitative rather than qualitative (Maroulis, 1980). However, since the rat adrenal cortex is unable to secrete significant amounts of either androgen or cortisol, and, since the synthesis of the major glucocorticoid, corticosterone, involves the mineralocorticoid (aldosterone) pathway (for review see Sandor, 1976), perhaps these arguments do not concern us.

1.2.3: Adrenocortical steroidogenesis

The adrenal cortex is the target tissue for the peptide hormone ACTH which is secreted by the anterior pituitary gland. The initial event in the action of ACTH is its binding to specific receptor sites on the membrane of the adrenal cell. This is followed by the activation of adenylate cyclase and the production of 3',5'-cyclic AMP (cAMP), which in turn activates protein kinase resulting in phosphorylation of the enzyme cholesterol ester hydrolase. The active cholesterol ester hydrolase catalyses the hydrolysis of stored cholesterol esters increasing the availability of free cholesterol to the mitochondria and its enzyme cytochrome P-450 side chain cleavage. In addition the binding of ACTH to its receptor also causes an increase in the synthesis of membrane phospholipids and the mobilisation of intracellular calcium, both of which may act as intracellular messengers for adrenal steroido-

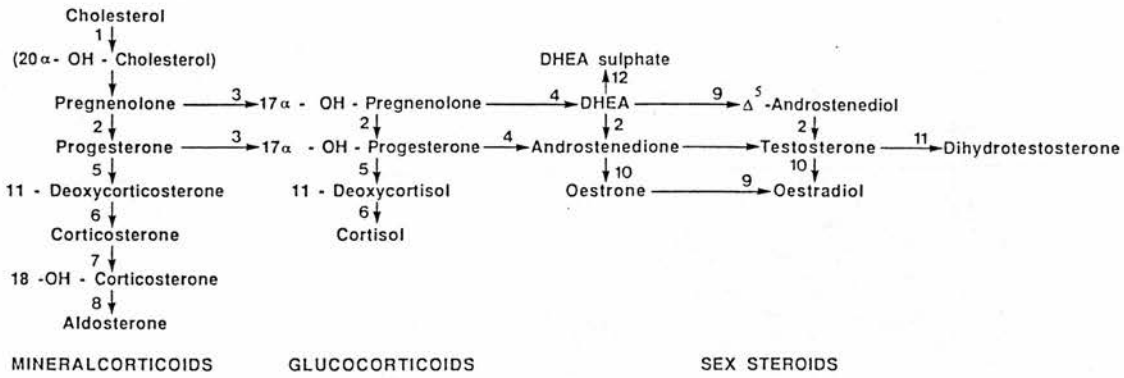


Figure 1.1: The major pathways of steroidogenesis in steroid secreting tissues. The enzymes involved in steroid synthesis are: (1) cholesterol desmolase complex; (2) 3β-hydroxysteroid dehydrogenase/isomerase complex; (3) 17α-hydroxylase; (4) 17,22-desmolase; (5) 21-hydroxylase; (6) 11β-hydroxylase; (7) 18-hydroxylase; (8) 11-hydroxysteroid dehydrogenase (9) 17β-hydroxysteroid oxidoreductase; (10) aromatase; (11) 5α-reductase; (12) sulphate conjugase (redrawn from Dewis, 1985).

genesis. The main corticosteroids secreted by the adrenal cortex are:

i) **Glucocorticoids** - cortisol and corticosterone.

ii) **Mineralocorticoids** - aldosterone.

iii) **Sex steroids** - a complex group consisting of the **androgens** (testosterone, dehydroepiandrosterone and its sulphate, and androstenedione), the **oestrogens** (oestradiol and oestrone) and the **progestagens** (progesterone). The possible biosynthetic pathways for these adrenocortical steroids are illustrated in figure 1.1. However, the principal steroids secreted by the rat adrenal cortex are 11-deoxycorticosterone, corticosterone, 18-hydroxycorticosterone and aldosterone (for review see Sandor, 1976). In the rat, steroidogenesis is therefore inhibited in the following pathways (Peron, 1968; Sandor, 1976):

i) **17,20-desmolase: 17α-hydroxypregnenelone ⟶ dehydroepiandrosterone ⟶ dehydroepiandrosterone sulphate.**

ii) **17,20-desmolase:** 17α-hydroxyprogesterone ⟶ androstenedione ⟶ testosterone.

iii) **21-hydroxylase:** 17α-hydroxyprogesterone ⟶ 11-deoxycortisol ⟶ cortisol.

The rat adrenal cortex does not appear to secrete significant amounts of either sex steroid or cortisol. The two main products of steroidogenesis in the rat are corticosterone as the glucocorticoid, and aldosterone as the mineralocorticoid. Nevertheless, it has been claimed that the rat adrenal may secrete cortisol (Vinson, 1976), 17α-hydroxyprogesterone (Vinson, 1976),

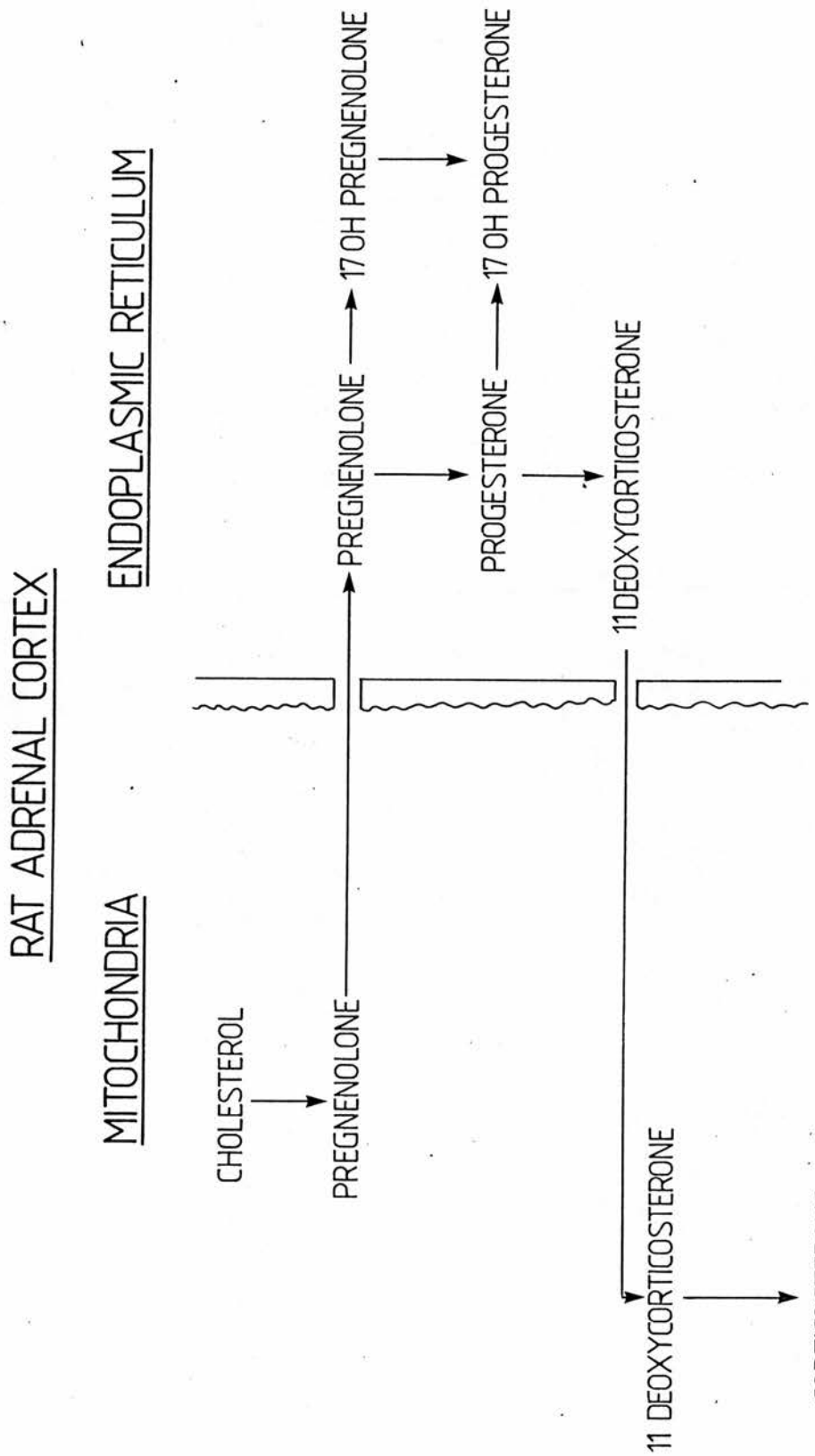


Figure 1.2: The pathways of steroidogenesis in the rat adrenal cortex (after Sandor, 1976).

androstenedione (Vinson, 1976; Bell, 1978) and testosterone (Vinson, 1976). However, evidence for 17-hydroxylation in the rat adrenal cortex remains controversial.

The pathways of rat adrenal steroidogenesis are illustrated in figure 1.2. It will be seen that steroidogenesis takes place in two compartments - the mitochondria and the cytoplasmic endoplasmic reticulum. The pathway from cholesterol through to corticosterone and aldosterone involves steroid hydroxylases which are different forms of cytochrome P-450. These enzymes catalyse the reaction whereby one atom of oxygen is added to the substrate, yielding an hydroxylated product, whilst the other atom of oxygen is used to form water. Cytochrome P-450 is a diverse and large family of enzymes bound to membranes of the mitochondria and endoplasmic reticulum. The conversion of cholesterol to pregnenelone (**cholesterol side-chain cleavage**) takes place in the mitochondria and, whilst the subject of this conversion has attracted some controversy, the details are not essential for the understanding of this thesis (for review see Simpson, 1979).

Following the mitochondrial synthesis of pregnenelone, its subsequent metabolism is carried out by the endoplasmic reticulum by the conversion of pregnenelone:

i) **17-hydroxylase:** pregnenelone ⟶ 17α-hydroxypregnenelone ⟶ (**3β-hydroxysteroid dehydrogenase plus isomerase**) 17α-hydroxy-progesterone.

ii) **3β-hydroxysteroid dehydrogenase plus isomerase:** pregnenelone ⟶ progesterone ⟶ (**17-hydroxylase**) 17α-

hydroxyprogesterone.

The final pathway of adrenal steroidogenesis involves the conversion of progesterone:

i) **21-hydroxylase:** progesterone ➟ 11-deoxycorticosterone ➟ (**11-hydroxylase**) corticosterone ➟ 18-hydroxycorticosterone ➟ (**18-hydroxylase**) aldosterone.

The activity of both the enzymes, **11-hydroxylase** and **18-hydroxylase**, resides in the mitochondrion and not in the endoplasmic reticulum, so that adrenal steroidogenesis switches back from the cytoplasm to the mitochondria for the synthesis of corticosterone and aldosterone.

Whether the intermediary metabolites of adrenocortical steroidogenesis have any function as hormones in their own right or whether they are unnecessary by-products of steroidogenesis is open to question (Short, 1960). For example, the adrenal secretion of androstenedione has been proposed as a prehormone in the human (Baird, 1968). Can a similar role be ascribed for adrenal steroid precursors in the male rat? The adrenocortical synthesis of precursor steroids such as progesterone and their subsequent secretion into the circulation may be the basis for direct adrenal ➟ gonad interaction, by providing the testis with metabolic substrates that can be used in the synthesis and secretion of testosterone.

For this to occur, it must be generally accepted that steroids can freely leave the adrenal gland, enter the circulation and enter the testis. There is a presumption that all steroids leave and

enter cells freely at identical rates (Sandor, 1976). This presumption has recently been tested by systematic experiments on the diffusional behaviour of different steroids through cell membranes (Giorgi, 1981). Velocity of passage of free steroids across plasma membranes is probably passive (simple diffusion) and the coefficient of diffusion is directly related to its partition coefficient in n-octanol. Thus, progesterone being a non-polar steroid with a high partition coefficient (124) in n-octanol/water, it has a high permeability coefficient (3.9×10^{-4} cm sec^{-1}) across plasma lipid membranes when compared with corticosterone (partition coefficient 46 and permeability coefficient 0.99×10^{-4} cm sec^{-1}) and will therefore leave adrenal cells and enter testicular cells with relative ease when compared with other steroids. Furthermore, not all steroids pass through membranes of subcellular organelles with the same ease (Vinson, 1969a; Vinson, 1969b; Vinson, 1970). Thus, the mitochondrion may act as a barrier to the passage of some steroids so that the administration of exogenous steroids from a biosynthetic pathway may not necessarily stimulate steroidogenesis.

1.2.4: Methodology for the study of adrenocortical steroidogenesis

Removal of adrenal tissue and manipulation under isolated conditions gives *in vitro* results which, with due caution, can be related to *in vivo* conditions. However, qualification of this statement is necessary, because results obtained *in vitro* may differ from those obtained *in vivo* (Vinson, 1971). The rat is an

ideal experimental animal providing an easily accessible and readily abundant supply of tissue but has the disadvantage of being so far removed from the human adrenal as to make comparisons difficult. For the practising physician in endocrinology, this barrier may at first appear insurmountable, yet this animal has provided a wealth of information on a wide and varying number of physiological conditions. This animal should at least help our understanding of the influence of adrenal gland upon testicular steroidogenesis.

The rat adrenal has been subjected to a wide variety of procedures involving either:

i) **static incubations** of isolated whole uncut adrenals (Stachenko, 1971), tissue slices (Saffran, 1953; Laplante, 1966), minced whole glands (Vinson, 1969a; Vinson 1969b), and isolated cells (Kloppenborg, 1968).

ii) **superfusion** of adrenals (Orti, 1965; Tait, 1967) and isolated cells (Lowry, 1974).

iii) **perfusion** of the isolated whole organ (Sibley, 1981).

Next to in vivo experiments and whole organ perfusions, continuous superfusion of isolated adrenal cells is probably the most physiologically relevant (Sandor, 1976).

In vitro cell suspensions are prepared by digestion of minced or quartered adrenal tissue with either trypsin or collagenase, followed by gentle mechanical dispersion achieved by repeated pipetting of the tissue (Haning, 1970). The resulting cell suspension of mixed adrenocortical cells produces low basal levels

of steroid that have increased sensitivity to ACTH when compared with other *in vivo* or *in vitro* techniques employed for bioassay (Kloppenborg, 1968). Both collagenase and trypsin have been used as the enzyme to digest adrenal tissue. When collagenase is employed, a crude preparation of the enzyme is used, because purified forms are largely ineffective (Kono, 1969). In contrast, trypsin is active in its purified form (Sayers, 1971), but has the disadvantage of being abrasive (Kono, 1971) and also requires reversal of its activity with trypsin inhibitor (Al-Dujaili, 1982) in order to prevent increased loss of cell surface receptors (Sayers, 1971). Furthermore, it has been recently demonstrated that trypsin (Raven, 1982) and its inhibitors (Pedersen, 1983; Raven, 1983) may have direct and profound effects upon adrenal steroidogenesis. For these reasons collagenase is preferred by many workers.

Following enzyme dispersion, zona reticularis and zona glomerulosa cells have a similar appearance by light microscopy. In contrast, zona fasciculata cells are larger (twice the diameter), with an increased cytoplasmic nuclear ratio and increased lipid content when compared with their reticularis counterparts (Bell, 1980). By electron microscopy, zona glomerulosa cells have tubular, zona fasciculata cells can be seen to have spherical and zona reticularis have mixed tubular/spherical mitochondrial cristae. Zona glomerulosa cells may be separated from zona fasciculata and reticularis cells by decapsulation of the whole adrenal gland prior to enzyme digestion. Isolated

populations of reticularis and fasciculata cells may then be separated by unit gravity sedimentation after digestion of the decapsulated gland. Using this method it has not been possible to demonstrate qualitative differences in steroid production by the two populations. However, quantitatively there is reduced 11-hydroxylation in the reticularis layer (Bell, 1980).

Dispersed adrenocortical cells are usually incubated in Krebs-Ringer buffer with glucose and albumen, either in static incubation or in a continuous superfusion system. Proponents of superfusion technology point out that in static incubations products remain in the medium and may alter secretion rates or be processed to abnormal end-products. Superfusion overcomes some of these problems and provides a system for the dynamic study of adrenal steroidogenesis. In general, steroid output is higher in the superfusion than the static system. This is in keeping with the observation that corticosterone is passively secreted and therefore sensitive to changes in the flow or volume of incubation medium (Sibley, 1980; Sibley, 1981).

Because these studies employ cells derived from enzymatically digested whole adrenal tissues, it is necessary to consider what effect enzymes of the adrenal medulla may have upon steroidogenesis of the adrenal cortex. Steroid hydroxylases have been demonstrated in phaeochromocytomas and in beef adrenal medulla slices (Carballeira, 1965) but studies performed in the rat show that adrenal medulla plays an insignificant role in steroid hydroxylation (Peron, 1968).

1.2.5: The hypothalamic-pituitary-adrenal axis

1.2.5.1.: Biochemistry and physiology of ACTH-related peptides

Anterior pituitary corticotrophin cells secrete ACTH as part of a precursor molecule, pro-opiomelanocortin (POMC) (Mains, 1977; Roberts, 1977). The sequence for bovine POMC consists of three peptides: ACTH, β-lipotrophin (β-LPH) and an N-terminal sequence, N-POMC, preceded by a signal peptide (Nakanishi, 1979). Human N-POMC consists of a peptide of 76 amino acids with an apparent molecular weight by gel filtration of 11200. In man, N-$POMC_{1-76}$ is the main secretory form of the N-terminal peptide (Estivariz, 1980) and the sequence N-$POMC_{79-109}$ is the connecting peptide between N-$POMC_{1-76}$ and ACTH (Seidah, 1981). N-POMC is secreted with ACTH and β-LPH under a wide range of physiological and pathological conditions.

The availability of purified N-POMC has made possible the production of specific radioimmunoassays against different parts of the sequence (Hope, 1981). All three peptides derived from POMC are secreted concomitantly into the circulation and their concentration in plasma increases after adrenalectomy and becomes undetectable after hypophysectomy (Lis, 1982). Concomitant secretion of these three products is stimulated by corticotrophin releasing factor (CRF) and inhibited by dexamethasone (Oki, 1982). Hope (1981) observed a close correlation between plasma concentrations of N-POMC and ACTH reflecting co-ordinated synthesis and secretion *in vivo*. Studies with rat pituitary corticotrophs *in vitro* indicate that the three peptides are secreted in equimolar

concentrations, reflecting post-translational cleavage of the parent molecule, POMC.

The amino acid sequence His-Phe-Arg-Trp encoded within the N-terminal portion of POMC is also shared by the melanotrophins α- and β-MSH encoded within the peptides ACTH and β-LPH respectively. Smaller peptides containing the sequences of N-POMC$_{51-61}$ (γ_1-MSH), N-POMC$_{51-62}$ (γ_2-MSH) and N-POMC$_{51-76}$ (γ_3-MSH) have been synthesised and used in the development of radioimmunoassays (Ling, 1979).

The species similarity of the three products of POMC suggests a related biological function. Although the biological actions of ACTH are well-established, at least in regard to the adrenal gland, no definite function for β-LPH and N-POMC has been determined. The observation that the first five residues of β-endorphin are common with the opiate peptide Met-enkephalin led to the discovery of its potent opiate-like activity (Li, 1977). However, despite intensive research on the matter, no clear role for an adrenal or extra-adrenal action of β-LPH/β-endorphin has emerged.

Interestingly, POMC is not only confined to the anterior pituitary but is also to be found in the central nervous system, placenta, and gastrointestinal tract (for review see Bardin, 1984). POMC peptides are also to be found in testicular Leydig cells and are processed in a similar manner to the anterior pituitary. Peptides derived from POMC are present in the testis during fetal life and following puberty, when testosterone secretion is maximal. Furthermore, the amount of testicular POMC increases with the administration of LH or hCG. Derivatives of POMC, ACTH or MSH

stimulate the growth of and accumulation of cAMP within Sertoli cells, whereas β-endorphin and/or another testicular opioid peptide inhibit Sertoli cell proliferation. The intratesticular administration of opiate antagonists inhibits testosterone secretion, suggesting that β-endorphin may stimulate testicular testosterone secretion. Thus POMC and its derivatives may represent a peptidergic system to be found in other steroid-secreting tissues, namely placenta, testis and adrenal (Bardin, 1984).

1.2.5.2: Neuroregulation of ACTH secretion

Anterior pituitary corticotroph cells receive blood from the hypothalamic-pituitary portal circulation. Secretion of POMC is under the direct control of the hypothalamic neurosecretory hormone known as CRF. This peptide is secreted into the portal circulation by neurones whose somas are localised in the mediobasal hypothalamus, with axons projecting into the median eminence. CRF is secreted in the vicinity of the portal capillaries in an episodic manner. There are three major factors involved in the secretion of ACTH:

i) **Circadian rhythm.** The existence of a circadian periodicity of plasma corticosteroid levels is well-documented. In the human peak levels of ACTH occur prior to or at the time of awakening and decline during the day, reaching a nadir during the late evening-early morning hours when episodic peaks of secretion are scarce or absent. This circadian rhythm is independent of

posture, persists with bed rest and persists in the absence of the adrenals, indicating an endogenous periodicity, synchronised by environmental phenomena such as the light-dark cycle (Krieger, 1978).

ii) **Negative feedback.** Corticosteroids exert negative feedback upon ACTH secretion. Two components of this feedback have been described: **short delay**, rate-sensitive, associated with the rate of rise of the plasma corticosteroid concentrations and possibly mediated by an effect on the release of CRF from the hypothalamus, and **delayed feedback**, level-sensitive, associated with the concentration of steroid reached in plasma, and probably mediated by an influence of corticosteroids on the rate of synthesis of CRF and/or the sensitivity of the corticotrophs to CRF (Jones, 1978).

iii) **Stress.** Negative feedback of corticosteroids upon the hypothalamus and pituitary can be overridden by severe stress such as burns, surgery or hypoglycaemia to secrete ACTH, and markedly elevated levels of both corticosteroids and ACTH are found in the circulation at these times (Nelson, 1980).

Although CRF was the first hypothalamic hormone to be demonstrated, attempts to isolate it in pure state were unsuccessful. One of the complicating factors has been the presence in posterior pituitary and median eminence of vasopressin (VP). This neurohypophyseal hormone *per se* has CRF-like activity. Recently, a 41-amino acid polypeptide has been isolated from ovine hypothalamus (oCRF). It is highly potent in stimulating secretion of ACTH by cultured

anterior pituitary cells, and synthetic peptides with similar structure display CRF activity *in vitro* and *in vivo* (Vale, 1981). Immunoneutralisation *in vivo* by exogenous anti-CRF antiserum inhibits the ACTH response to exogenous CRF and stress, and markedly inhibits ACTH levels in adrenalectomised rats (Rivier, 1982). Moreover, the activity of the putative 41-residue CRF *in vitro* is potentiated by arginine-VP (Turkelson, 1982). However, the stimulation of CRF by stress seems to be independent of the potentiating effect of VP, as antagonists of the neurohypophyseal nonapeptide do not abolish ACTH response to psychological stress (Mormede, 1983). It is thus likely that VP may be a biological co-factor of CRF for certain types of response only.

The activity of the CRF-producing cells is modulated by the nerve inputs from other areas of the brain (for review see Weiner, 1978). Noradrenergic nerves are possibly involved in a negative control of CRF secretion (Lancranjan, 1979), whereas serotoninergic neurones may stimulate the release of CRF (Fuller, 1981); a role for histamine in the control of CRF is more controversial. γ-Aminobutyric acid (GABA) may have an inhibitory action on CRF secretion, and this has been used in the treatment of patients with Nelson's syndrome with drug agonists of GABA (Dornhorst, 1983). Opiate peptides are also possible modulators of CRF secretion; the opiate agonist peptide D-Ala^2-MePhe^4-Met-enkephalin-O-ol (DAMME) inhibits the secretion of ACTH (Delitala, 1981), and naloxone causes an elevation in serum cortisol in normal human subjects, suggesting that a tonic inhibition on ACTH is exerted by endogenous

opiates (for review see Grossman, 1983).

1.2.5.3: Actions of ACTH, N-POMC and β-LPH on the adrenal gland

ACTH was known for many years before details of its precursor were elucidated. It is accepted at present that ACTH is the pituitary hormone responsible for the control of steroidogenesis by the adrenal cortex. A great deal of information regarding its structure, biological acitivity, structure-function relationships, assays and mechanism of action has been published (for review, see Rees, 1979). ACTH exerts many actions upon the adrenal cortex, including an increase in blood flow, production of hypertrophy, depletion of ascorbic acid, depletion of cholesterol esters and the synthesis of corticosteroids. It also stimulates the uptake of lipoprotein-bound cholesterol and induces a number of adrenal enzymes.

The initial event in the action of ACTH is its binding to specific receptor sites on the membrane of the adrenal cell. This is followed by the activation of the enzyme adenylate cyclase and the production of cAMP. Increased intracellular cAMP levels cause an activation of protein kinase activity which in turn is believed to result in phosphorylation of the enzyme cholesterol ester hydrolase, converting it from an inactive to an active form (Trzeciak, 1973; Beckett, 1977; Naghshineh, 1978). The active cholesterol ester hydrolase catalyses the hydrolysis of stored cholesterol esters in the lipid droplets and thereby increases the amount of free cholesterol available to the mitochondria resulting

in an increase in cholesterol binding to cytochrome P-450 **side-chain cleavage enzyme.** In the adrenal cortex, as in other steroid secreting tissues, the rate-limiting step in steroid hormone biosynthesis is the conversion of cholesterol ⟶ pregnenelone by cytochrome P-450 **side-chain cleavage** enzyme (for reviews see Schulster, 1976; Simpson, 1979).

The binding of ACTH to its receptor also causes a rapid increase in membrane phospholipids (Farese, 1980; Farese, 1983) resulting in the synthesis of phosphatidyl inositol monophosphate (PI), biphosphate (PI_2) and triphosphates (PI_3). Both PI_2 and PI_3 stimulate cytochrome P-450 **side-chain cleavage enzyme** in a similar manner to ACTH (Farese, 1979) resulting in the formation of pregnenelone (Farese, 1981). Other phospholipids may be released by the binding of ACTH to its receptor. Activation of phospholipase A_2 results in the release of arachidonic acid from the cell membrane (Schrey, 1979).

Calcium is probably a further messenger in ACTH-mediated steroidogenesis. Not only is calcium involved in the adenyl cyclase system and phospholipid metabolism, but it may act as a messenger in its own right. It has been known for some time that calcium can stimulate steroid hydroxylations in the adrenal mitochondria (Peron, 1968) and in particular stimulates cytochrome P-450 **side-chain cleavage enzyme** (Simpson, 1975). Furthermore, calcium-induced stimulation of pregnenelone formation is caused by a redistribution of cholesterol within the mitochondrial membranes, increasing the proportion of cholesterol bound to P-450 **side-chain**

cleavage enzyme. The mobilisation of calcium from extracellular to intracellular stores may be controlled by the metabolites of the phosphatidyl inositol pathway (Michell, 1975) and the intracellular content of calcium is regulated by the intracellular actvity of calmodulin (Hall, 1981).

Since the discovery of POMC there has naturally been a search for a possible biological role for the N-terminal peptide. Indeed, N-POMC derived from a mouse pituitary tumour cell line has been shown to potentiate ACTH-induced steroidogenesis by isolated rat adrenocortical cells (Pedersen, 1980a), but it was necessary to trypsinise the peptide for it to be effective. However, trypsin and its inibitors may directly influence adrenal steroidogenesis *in vitro* (Raven, 1982; Raven, 1983; Pedersen, 1983), providing one explanation for this intriguing observation. Alternatively, the biological activity of N-POMC may reside in a smaller part of the fragment that is released by trypsinisation. This may be in the region of γ-MSH. Synthetic γ_3-MSH is also capable of potentiating ACTH-stimulated adrenocortical steroidogenesis and stimulating the activity of the adrenocortical enzyme, **cholesterol ester hydrolase** (Pedersen, 1980b). In contrast, it has been shown that Lys-γ_3-MSH failed to stimulate an increase in cAMP or membrane phospholipids (Farese, 1983). Other authors have also described the presence of ACTH-potentiating factors in peptides of high molecular weight obtained from rat anterior pituitary extracts (Iida, 1981). Human N-POMC significantly potentiates ACTH-stimulated steroidogenesis *in vitro* using superfused trypsinised isolated rat adrenocortical

cells (Al-Dujaili, 1981a). The mechanism of action of potentiation appears to require the presence of intact transcriptional mechanisms, since the effect of N-POMC is abolished by inhibitors such as actinomycin D and mithramycin (Al-Dujaili, 1982).

A further role has been suggested for N-POMC-derived peptides (Estivariz, 1982). Some fragments (N-POMC$_{1-28}$) derived by limited proteolysis from N-POMC are potent stimulators of adrenal DNA synthesis *in vitro*, and of adrenal mitosis *in vivo*. Furthermore, the administration *in vivo* of antisera directed against the extreme N-terminal portion of N-POMC$_{1-28}$ and the mid-portion of N-POMC$_{1-74}$ both prevented compensatory hyperplasia of the adrenal gland remaining after unilateral adrenalectomy (Silas, 1983). Thus N-POMC may be responsible, in part, for the regulation of adrenocortical cell growth. The activation of **cholesterol ester hydrolase** activity, originally observed by Pedersen (1980a) is also mediated by another region, N-POMC$_{57-76}$.

Little is known about the adrenal action of β-LPH/β-endorphin. However, it has long been thought that a non-ACTH pituitary factor may play a role in the regulation of aldosterone during sodium depletion. Indeed, β-LPH has been shown to stimulate aldosterone secretion by isolated rat adrenal zona glomerulosa cells (Matsuoka, 1981). β-MSH was equally potent as β-LPH but concentrations of the peptides were pharmacological rather than physiological. β-Endorphin failed to stimulate aldosterone secretion, in contrast to that reported previously (Shenker, 1979). Sodium depletion enhances the sensitivity of adrenal zona glomerulosa cells to β-

MSH (Yamakado, 1982) and aldosterone concentrations obtained by maximal doses of β-MSH were similar to those achieved by maximal doses of angiotensin II. Thus β-MSH and relate peptides may play a role in the regulation of aldosterone secretion in the rat, particularly in the salt-depleted state.

1.3: The Testis

1.3.1: Historical perspectives

The testis has long been recognised as the organ responsible for fertility in men. Indeed, the word 'testify' has its origins in the Roman practice of refusing a testimony to stand unless the man could prove that his testicles were intact.

It was Hamen in 1677 who first discovered spermatozoa but it was not until 1841 that these cells were found to originate from the testis and 1865 that they were proved to be involved in fertilisation. The structure of the testis was described in 1668, the Leydig cell was named after its discoverer, von Leydig, in 1850 and the Sertoli cell was named for its discoverer, by the German scientist von Ebner, in 1888. In 1903 the Leydig cell was postulated as the site of synthesis of many factors responsible for the development of male characteristics. In the 1930s the androgens androstenedione and testosterone were purified and the separate pituitary products Luteinising Hormone (LH) and Follicle Stimulating Hormone (FSH) identified. Inhibin was postulated as a testicular product to regulate anterior pituitary FSH secretion in 1932 (source, Bartlett, 1985).

1.3.2: Testicular structure and function

The mammalian testis is separated into two major compartments - the avascular seminiferous tubules (82% of testicular mass) and the vascularised interstitial space (16% of testicular mass) (Mori, 1980).

The seminiferous tubules are the site of sperm production and histology of its epithelium reveals a highly organised cycle of spermatogenesis (Leblond, 1952). A detailed discussion of spermatogenesis is not within the scope of this thesis.

The interstitial spaces of the testis contain Leydig cells (3% of testicular mass), macrophages, fibroblasts, lymphatics and interstitial fluid (Christensen, 1975). In the rat the volume of this fluid increases with age and following hCG injection, reflecting changes in the permeability of testicular blood vessels (Sharpe 1977; Setchell, 1981; Sharpe, 1983a; for review see Sharpe, 1984). The compartmentalisation of the testis into seminiferous tubules and the interstitial spaces is not only anatomical but also functional, isolating the seminiferous epithelium either by the layer of myoid cells that surrounds the tubules (Dym, 1970) or by the Sertoli cells, linked together by tight junctions (Setchell, 1980a). This functional barrier is thus able to maintain the hormonal environment of the seminiferous tubules that is required for normal spermatogenesis. It is thought that lipid soluble materials enter the seminiferous tubules by traversing the Sertoli cells (Setchell, 1980a), whereas water soluble materials are prevented from entering by the spaces between the Sertoli cells

because of the tight junctions. The Sertoli cells also secrete an androgen-binding protein (ABP) in response to FSH (Fritz, 1975) and androgens (Elkington, 1975). ABP has a high affinity for testosterone diffusing into the tubules from the interstitial spaces and binding of the androgen may prevent its further metabolism, maintaining a high concentration of androgen within the tubule (Dorrington, 1980).

The presence of the Sertoli cell barrier has major implications for the physiology of the testis. It is of great interest that this barrier appears during pubertal development (approximately 30-50 days of age) (Setchell, 1980b), and is associated with the parallel development of a barrier in the testicular capillaries that excludes certain dyes from access to the interstitial space. The development of a barrier may, therefore, account for the inability of the testis in vitro to convert exogenous progesterone to androgen during puberty, whereas this in vitro conversion is easily achieved before and after puberty (Steinberger, 1968).

The Leydig cell is the source of testosterone production by the testis. In the rat, there are two generations of Leydig cells with different morphological appearances. The first generation, or fetal Leydig cells, appears in utero as early as the fifteenth day of gestation and disappears after birth. The second generation, or adult Leydig cells, appears in puberty in association with a simultaneous reduction of fetal cells (Clegg, 1966; Lording, 1972; Picon, 1980). The adult population is derived by differentiation from precursor cells as well as directly from dividing differen-

tiated cells (Hooker, 1970). The increase in the population of adult Leydig cells is accompanied by a progressive increase in plasma testosterone concentrations (Lescoat, 1982). The majority of the enzymes involved in the biosynthetic pathway of testosterone reach their peak activity at 30-35 days of age (Inano, 1967; Payne, 1977). The exception is the enzyme 17β-hydroxysteroid oxido-reductase, the final enzyme in the pathway, which reaches its peak activity slightly later. In association with the activity of these enzymes there is a significant surge in plasma LH levels from day 30 to 35 and plasma testosterone concentrations (Lescoat, 1982; see Sharpe, 1982 for review), suggesting that the development of the adult population of Leydig cells is under the control of the anterior pituitary. Indeed, the development of these cells can be inhibited by hypophysectomy (Hooker, 1970). Whilst LH is the major hormone to control Leydig cell function, elevated plasma FSH levels during puberty also correlate with the development of Leydig cells (Sharpe, 1982). Since Leydig cells do not possess FSH-receptors, it is possible that the FSH-dependent Sertoli cell may influence the development of adult Leydig cells.

1.3.3: Testicular steroidogenesis

The Leydig cell is the target tissue for the peptide hormone LH which is secreted by the anterior pituitary. Leydig cells synthesise and secrete testosterone involving biosynthesis from acetate via cholesterol (Hall, 1969). The rate-limiting step in testosterone biosynthesis, as in other steroid-secreting tissues,

is the conversion of cholesterol ➟ pregnenelone by side-chain cleavage. The major biosynthetic pathways of steroid-secreting tissues are illustrated in figure 1.1. However, the principle steroids secreted by rat Leydig cells are progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone (Chubb, 1979b). Thus in the rat testis there is little activity in the pathways:

i) **21-hydroxylase:** progesterone ➟ 11-deoxycorticosterone and 17 -hydoxyprogesterone ➟ 11-deoxycortisol.

ii) **7,20-desmolase:** 17α-hydroxypregnenelone ➟ dehydroepiandrosterone ➟ dehydroepiandrosterone sulphate.

The pathways for testicular steroidogenesis in the rat are illustrated in figure 1.3. It will be seen that steroidogenesis takes place in two compartments - the mitochondria and the cytoplasmic endoplasmic reticulum. The pathway of steroidogenesis from cholesterol to testosterone involves cytochrome P450-dependent steroid hydroxylases. The conversion of cholesterol to pregnenelone (**side-chain cleavage**) takes place in the mitochondria but the subsequent metabolism of pregnenelone is carried out in the endoplasmic reticulum:

i) **17-hydroxylase:** pregnenelone ➟ 17α-hydroxypregnenelone ➟ (**3β-hydroxysteroid dehydrogenase plus isomerase**) 17α-hydroxyprogesterone.

ii) **3β-hydroxysteroid dehydrogenase plus isomerase:** pregnenelone ➟ progesterone ➟ (**17-hydroxylase**) 17α-hydroxyprogesterone.

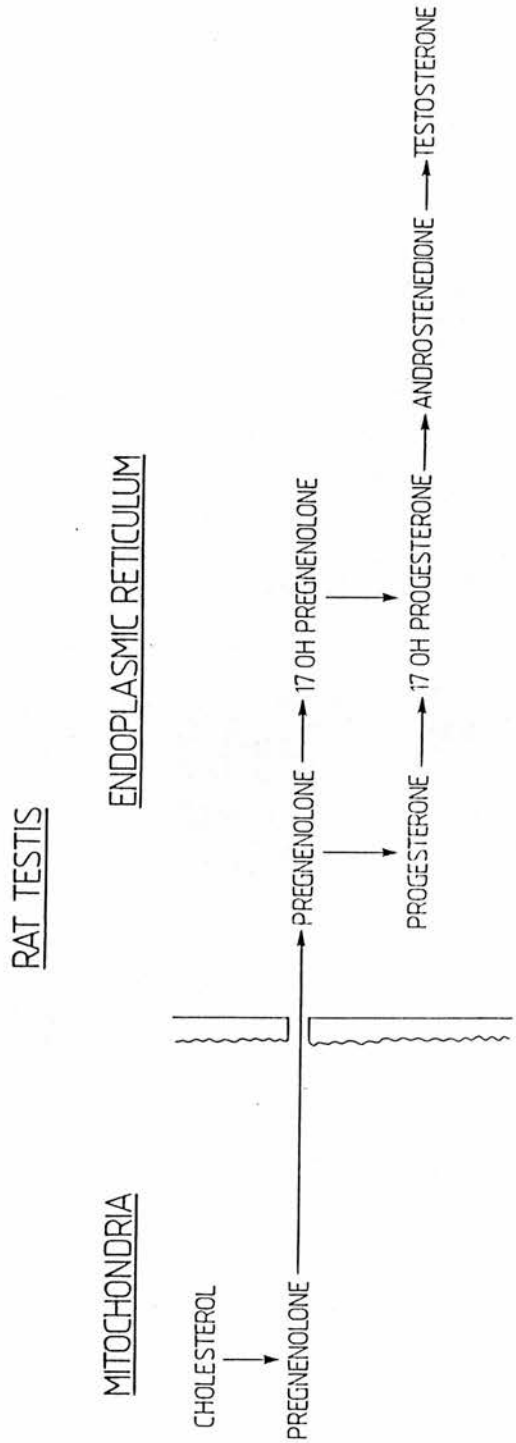


Figure 1.3: The pathways of steroidogenesis in the rat testis (after Chubb, 1979b).

Unlike the adrenal, steroidogenesis remains in the endoplasmic reticulum without returning to the mitochondria. This factor may therefore be of some importance in adrenal ➟ gonad interactions, permitting the direct testicular utilisation of any steroid precursors that are secreted by the adrenal gland into the circulation.

From 17α-hydroxyprogesterone, the final common path in testosterone biosynthesis is ➟ (**17,20 desmolase**) androstenedione ➟ (**17 hydroxysteroid oxoreductase**) testosterone.

1.3.4: Methodology for the study of testicular steroidogenesis

Like the adrenal gland, removal of testicular tissue and manipulation under isolated conditions gives *in vitro* results which, with due caution, can be related to *in vivo* conditions. The rat testis has been subjected to a wide variety of procedures involving either:

i) **static incubations** of isolated whole testis, isolated rat Leydig tumour cells (Browning, 1983) and freshly purified isolated cells (Mendelson, 1975a; Mendelson 1975b).

ii) **superfusion** of testes, isolated rat Leydig tumour cells (Segaloff, 1981) and freshly isolated testicular cells (Davies T F, 1981; Kühn-Velten, 1982).

iii) **perfusion** of the whole organ either *in vitro* (Chubb, 1979a) or *in situ* (Frederik, 1973; Baker, 1977).

Experiments with perfusion of the whole organ have the disadvantages of limited tissue viability and the development of

vascular disturbances, resulting in oedema and underperfusion (Baker, 1980). Next to *in vivo* experimentation, continuous superfusion is, therefore, probably the most suitable for *in vitro* studies.

In vitro cell suspensions of testicular tissue are prepared by digestion of whole decapsulated testes with collagenase (Davies, 1979), without mechanical dispersion. Dispersed testicular cells are usually incubated in Krebs-Ringer buffer with glucose and albumen, either in static incubation or in a continuous superfusion system. As with isolated adrenal cells, proponents of superfusion technology point out that in static incubations products remain in the medium and may alter secretion rates or be processed to abnormal end-products. Superfusion overcomes these problems and provides a system for the dynamic study of testicular steroidogenesis. Static incubations are limited to short time intervals and isolated cells are better preserved in the superfusion system for experiments of two hours or more. In the superfusion system, a suspension of either isolated-mixed testicular (Davies T F, 1981) or Leydig tumour cells (Segaloff, 1981) produces low basal levels of steroid hormone that respond to both ovine-LH (oLH, over the dose range 1-1000 ng/ml) or hCG (1 ng/ml). There are substantial differences in the testosterone response by isolated cells to oLH when compared with hCG. The response curve to a pulse of oLH is Gaussian in its distribution and the duration of its effect is confined to the period that oLH remains in the superfusate. In contrast, the response to a pulse of hCG is skewed to the right,

persisting after the superfusion of hCG through the system. Repeated stimulation of the isolated cells with either oLH or 8-Br-cAMP leads to diminished responsiveness. This presumably reflects either a loss of cell viability or 'down-regulation' of LH receptors that may occur in the target organ following repeated trophic stimuli.

Following collagenase dispersion, functional and morphological studies of isolated testicular cells have been performed. When viewed under the phase contrast microscope they are characterised by a yellowish appearance with a granular texture and surrounded by a bright yellow halo (Gale, 1982). The active population of collagenase-dispersed rat Leydig cells may be composed of two or more subpopulations of cells with different densities and different biological activities when purified by Ficoll (Janszen, 1976) and metrizamide (Payne, 1980b). However, other studies on the population of collagenase-dispersed cells, whilst demonstrating two subpopulations of Leydig cells, have shown no difference in morphology or biological activity when purified using metrizamide with (Aquilano, 1984) or without centrifugal elutration (Payne, 1980a). It is therefore difficult to know whether to attribute these findings to differences in methodology or to real differences in Leydig cell population.

1.3.5: The hypothalamic-pituitary-testicular axis

1.3.5.1: Physiology of gonadotrophin secretion

The anterior pituitary secretion of gonadotrophins is at any

time influenced by an interplay between gonadal steroids and the hypothalamic secretion of gonadotrophin-releasing hormone (GnRH). The influence of sex steroids is easily demonstrated by the increased secretion of both gonadotrophins in response to castration. However, the actions of both these hormones are separated within the testis, LH acting upon the Leydig cell and FSH upon the seminiferous epithelium (Greep, 1936; Dorrington, 1974). Testosterone is the major signal in the peripheral circulation capable of modulating the anterior pituitary secretion of LH in the male rat (Kingsley, 1973; Drouin, 1976; Giguere, 1981), whereas the major peripheral signal regulating the anterior pituitary secretion of FSH is inhibin (Main, 1979; Baker 1983; Steinberger 1983).

1.3.5.2: Neuroregulation of gonadotrophin secretion

During the course of studies of LH secretion in castrated primates, marked variations in the levels of LH were observed. On making measurements at more frequent intervals the secretion of LH was discovered to be pulsatile (Diershke, 1970) and this finding was later extended to the experimental male rat (Gay, 1972; Kalra 1977; Ellis, 1982). Low levels of pulsatile secretion are established early in the juvenile period and well established by the onset of puberty (Andrews, 1981), but with advancing age there is progressive loss of periodicity (Meites, 1982). Pulsatile secretion of LH occurs at regular intervals of 50-70 minutes. Whilst there is little evidence of coupling of pulsatile LH

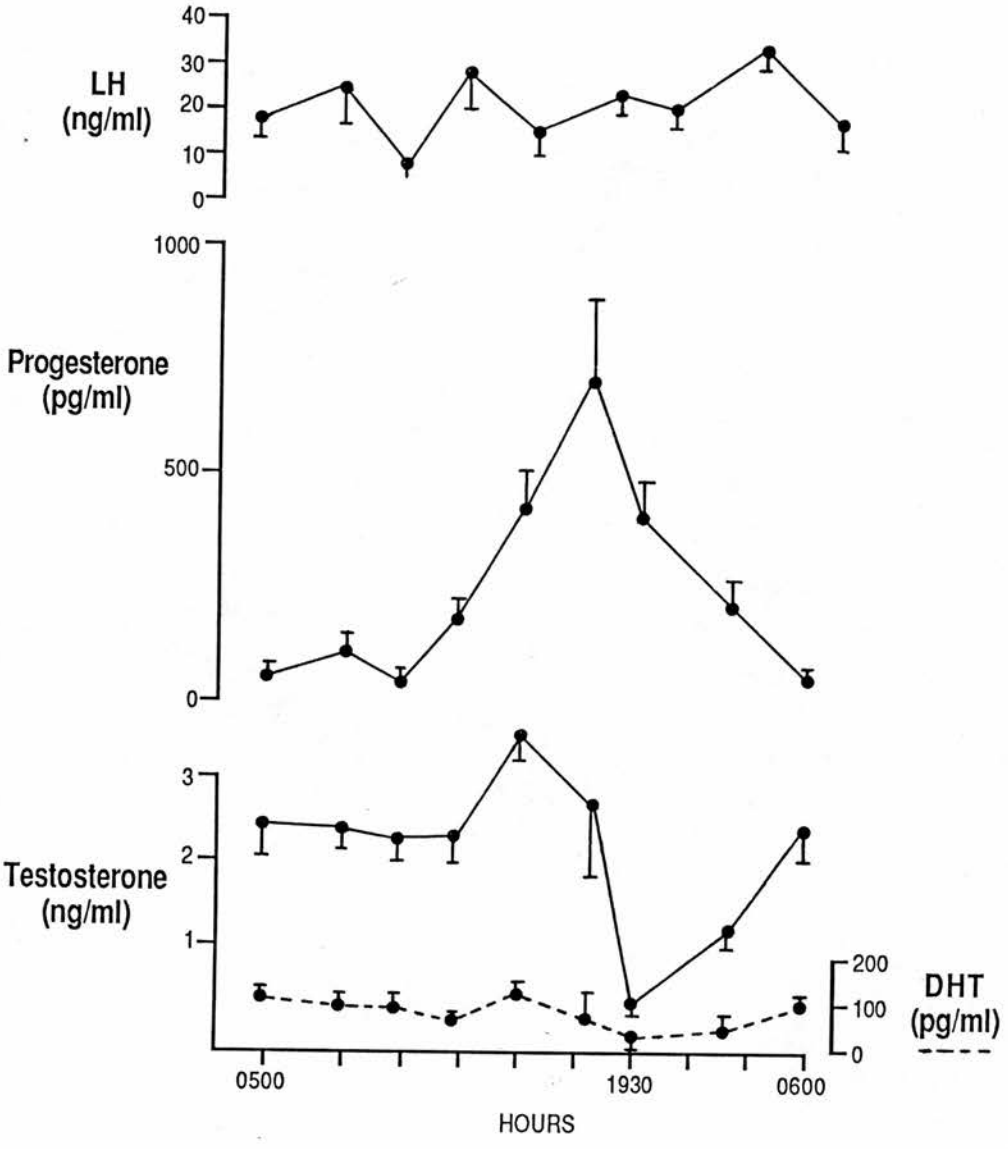


Figure 1.4: Circadian rhythm of serum levels of LH, progesterone, dihydrotestostone and testosterone in the male rat (from Kalra, 1977).

secretion with other circadian rhythms, the circadian secretion of progesterone appears to be related to a biphasic secretion of testosterone by the testis (Fig 1.4, redrawn from Kalra, 1977). The pulsatile release of LH is presumably based on an episodic secretion of GnRH by the hypothalamus (pulse generator) (for review see Kalra, 1983; Belchetz, 1983). The inescapable conclusion from experiments by Knobil (1980) is that GnRH acts in a permissive fashion to prime anterior pituitary gonadotrophs. Anterior pituitary secretion of LH is subject to various factors including the hypothalamic secretion of GnRH as well as the influences of sex-steroids.

i) **Pulse generator.** GnRH activity is unevenly distributed throughout the rat brain. Concentrations are highest in the median eminence with lower levels in the organum vasculosum of lamina terminalis (OVLT), arcuate nucleus (ARC), reterochiasmatic area (RCA), suprachiasmatic nucleus (SCN) and supraoptic nucleus (McCann, 1960; Harris, 1961; for review see Kalra, 1983). Studies have been extended to localise GnRH activity to specific GnRH-secreting neurones. These neurones converge from the brain into the RCA, traversing to the premamillary zone and terminating in the primary portal plexus of the anterior pituitary. The densest accumulation of GnRH-secreting neurones is to be found in the median eminence. The origin of these fibres has been traced by deafferentiation experiments, lesion studies as well as pharmacological studies to the SCN, mediobasal hypothalamus as well as areas outside the hypothalamus. Thus the pulse generator appears

to be a complex anatomical network of GnRH-secreting neurones that innervate the median eminence and receive a variety of neurophysiological and neuropharmacological signals from within and without the hypothalamus. Dopamine, catecholamines, serotonin, acetylcholine and opioid peptides are all important neurotransmitters that modulate the pulse generator. However, the precise mechanisms involved in the generation of episodic GnRH secretion are poorly understood. According to one hypothesis, the clock-like precision of GnRH secretion resides within the peptidergic neurones, with modulation of the frequency and amplitude of secretion of GnRH by other neural and hormonal inputs. Alternatively, periodic discharge of GnRH may arise outside GnRH-secreting neurones in a network of nerves that imposes its own periodicity on GnRH secretion (for review see Kalra, 1983).

ii) **Negative feedback.** Testosterone is the major signal in the peripheral circulation capable of modulating the anterior pituitary secretion of LH in the male rat, acting both directly at the level of anterior pituitary gonadotrophs (Kingsley, 1973; Drouin, 1976; Giguere, 1981) and indirectly at the level of the hypothalamus (Damassa, 1976; Clayton 1982; Kalra 1983). There is a daily rhythm of the testicular secretion of testosterone upon which wide fluctuations may be superimposed (Bartke, 1973; Kinson, 1973; Kalra, 1977; Mock, 1978; Kalra, 1979; Keating, 1979). Within hours of castration, there is a rapid rise in plasma LH levels associated with an increased frequency of LH secretion (Nansel, 1979; Kalra, 1983) and a later increase in the pulse amplitude.

Androgen replacement returns the pattern of LH secretion to that of intact rats. Testosterone replacement appears to reduce pulse amplitude rather than pulse frequency (Nansel, 1979) unless androgen replacement is administered prior to castration, in which case both pulse amplitude and frequency are reduced (Steiner, 1982). Androgens, therefore, modulate the response of anterior pituitary gonadotrophs to the periodic signal generated by the hypothalamus. This is supported by suppression of the LH-response to GnRH by anterior pituitary cells in vitro following pretreatment with testosterone (Drouin, 1976), and enhancement of this response by anterior pituitary cells obtained from castrated rats (O'Connor, 1980). Androgens may also act in vitro by reducing the number of GnRH receptors (Giguere, 1981).

The role of androgens in the regulation of the hypothalamic secretion of GnRH is less clear. Whilst testosterone and its metabolites increase and castration decreases the median eminence content of GnRH, there is no evidence that these changes influence the amount of GnRH secreted by the hypothalamus (Kalra, 1983).

In addition to testosterone, its metabolites - dihydrotestosterone and oestradiol - circulate in the blood in sufficient concentrations to act in their own right or in concert with other androgens on the hypothalamo-pituitary axis (Damassa, 1976; Kalra, 1977; Kalra, 1981). However, evidence that progesterone influences the anterior pituitary secretion of LH is equivocal. The administration of progesterone to both intact and castrated male rats either reduces or has little influence upon plasma LH concentrat-

ions (Kingsley, 1973; Brown-Grant, 1974; Aiyer, 1976; Fink, 1976).

Inhibin is the major signal in the peripheral circulation capable of modulating the anterior pituitary secretion of FSH, acting directly on the pituitary (Baker, 1983) as well as at the level of the hypothalamus to modulate the secretion of GnRH (Labrie, 1978).

iii) **Stress.** The diverse effects of stress upon the anterior pituitary, adrenal and gonadal secretion of hormones is well known (for review see Andrews, 1977). In particular, stress in various forms including animal handling, blood collection, novel environments and anaesthesia may have profound and adverse effects on the hypothalamo-pituitary-gonadal axis in rats.

In male rats, the **acute response** to stress provokes a marked increase in plasma LH concentrations (Dunn, 1972; Krulich, 1974; Euker, 1975; Turpen, 1976; Lorenzen, 1980; Lescoat, 1984) as well as an associated rise in plasma testosterone concentrations (Kniewald, 1971; Kamel, 1978; Lescoat, 1984). The initial rise in gonadotrophin secretion observed during this acute phase is also associated with increased secretion of ACTH and prolactin from the anterior pituitary and increased secretion of corticosterone and progesterone from the adrenal gland (Euker, 1975; Lorenzen, 1980; Lescoat, 1984).

However, this acute response is short-lived, lasting approximately 30-90 minutes, and is then followed by a **chronic phase**, characterised by profound suppression of secretion of both gonadotrophin (Lorenzen, 1980; Ringstrom, 1984) and testosterone

(Free, 1973; Stahl, 1984). The duration of this chronic phase usually lasts from 4 to 48 hours, or more and presumably reflects adaptation of the hypothalamus (GnRH) or pituitary (LH) to stress.

GnRH is not only confined to the hypothalamus but is to be found in a wide range of tissues including the adrenal glands, pancreas, pineal, placenta, ovary and testis (Sharpe, 1984). GnRH acts directly on the testis to modulate LH-induced steroidogenesis, and the testicular secretion of GnRH by Sertoli cells may be an important local regulator of testosterone production by the testis (for review see Sharpe, 1984).

1.3.5.3: Actions of gonadotrophin on testicular steroidogenesis

LH was first purified from the anterior pituitary in the 1930s and its availability provided evidence that LH directly stimulated Leydig cells by maintaining normal Leydig cell morphology after hypophysectomy (Greep, 1936). However, it was not until much later that it was shown that the rat Leydig cell was controlled by the anterior pituitary secretion of LH (Mancini, 1967). Both LH and hCG stimulate testosterone production in freshly dispersed rat Leydig cells (Mendelson, 1975a) and in Leydig cells in primary culture (Hsueh, 1980). Increased steroidogenesis is mediated by the binding of LH to specific receptors, activation of adenyl cyclase and increased formation of cAMP (Catt, 1978). The subsequent cAMP-mediated events result in an increase in testicular steroidogenesis. The stimulation of testosterone synthesis by the administration of hCG to freshly dispersed Leydig

cells is dependent on both RNA and protein synthesis (Mendelson, 1975b), suggesting that the stimulation of steroidogenesis may be mediated by increased synthesis of a protein that controls the enzymes of the steroidogenic pathway. Cholesterol is mobilised from lipid droplets in the cytoplasm by the enzyme **cholesterol ester hydrolase** and it is subsequently converted in the mitochondria by cytochrome P-450 (**side-chain cleavage enzyme**) to pregnenelone. Administration of LH or hCG to hypophysectomised rats restores the activity of the cytochrome P-450 **side-chain cleavage enzyme** (Menon, 1967), **3β-hydroxysteroid dehydrogenase** activity (Samuels, 1956) and testosterone synthesis (Purvis, 1981).

GnRH also appears to directly influence testicular steroidogenesis. Agonists of GnRH bind specifically to purified rat Leydig cells (Hunter, 1982), stimulating steroidogenesis in the short term (Hunter, 1982; Sharpe, 1982) but inhibiting LH-mediated steroidogenesis in the longer term (Hunter, 1982; Massicotte, 1980). However, GnRH agonists do not mediate their effect by using cAMP as the intracellular messenger (Sullivan, 1984) but probably stimulate the mobilisation of intracellular calcium stores (Sullivan, 1983a; Sullivan, 1983b; Cooke, 1984). It has been recognised for some time that calcium is required for steroidogenesis (Birmingham, 1953; Janszen, 1976), specifically by the enzyme responsible for side chain cleavage (Hall, 1981). The metabolism of arachidonic acids from membrane phospholipids by the enzyme phospholipase A_2 is also a calcium-dependent system. Calcium ionophores potentiate arachidonic acid metabolism and stimulate LH-induced testicular

steroidogenesis (Sullivan, 1984), whereas inhibitors of arachidonic acid metabolism inhibit LH-induced testicular steroidogenesis (Dix, 1984). Other phospholipids have been implicated in steroidogenesis (Farese, 1980) and the metabolism of phosphatidyl inositol may control calcium entry into the cell (Michell, 1975). Thus GnRH may act by stimulating the metabolism of membrane phospholipids resulting in an influx of calcium, stimulating cholesterol transport into mitochondria and the activity of its **side-chain cleavage enzyme** (Cooke, 1984).

In contrast, FSH has little activity in testicular steroidogenesis. One of the major effects of FSH is to stimulate the production of ABP by the Sertoli cells although the primary regulator of this protein is probably testosterone (Tindall, 1976). Both FSH and LH are necessary for the successful initiation of spermatogenesis in immature and hypophysectomised animals (Lostroh, 1963; Steinberger, 1971; Go, 1971). However, FSH is not absolutely essential for the maintenance of spermatogenesis once this has been successfully established (Davies A G, 1981).

1.4: Adrenal ⟶ gonad interactions

The possibilities that the adrenal gland may influence the gonad in both sexes has been the topic of intense speculation (Kime, 1981). In the male rat the major pathways of influence appear to be the direct influence of adrenal corticosteroids on the testis and the indirect influence of these steroids on the anterior pituitary secretion of gonadotrophins.

1.4.1: Adrenal ➡ gonad interactions in vivo: the effect of adrenalectomy on gonadal function in the male rat

There have been several studies investigating the effects of adrenalectomy on male gonadal function in vivo. These studies can be divided into **acute** and **chronic** effects:

i) In **acute studies** (Kniewald, 1971) bilateral adrenalectomy, performed in adult rats, results in a profound decline in plasma testosterone levels when compared with those levels following unilateral adrenalectomy, when the remaining adrenal gland underwent compensatory hyperplasia (Fig 1.5). In contrast, castration resulted in an initial rise in plasma testosterone concentrations at one hour before declining to undetectable levels at 64 hours. These results were interpreted as indicating that either the adrenal gland is capable of secreting significant amounts of testosterone or that the adrenal gland contributes to the biosynthesis of testosterone by providing the testis with an essential precursor; progesterone was tentatively proposed as such a precursor. This is supported by the observation that the initial rise in plasma testosterone concentrations following castration is abolished when bilateral adrenalectomy is combined with castration. Unfortunately, there are difficulties in the interpretation of this study. It is not correct to compare plasma testosterone levels for up to 12 hours following bilateral adrenalectomy with those levels at 64 hours following unilateral adrenalectomy. Animals undergoing unilateral adrenalectomy were presumably left for this longer period to allow the remaining adrenal to compensate. This

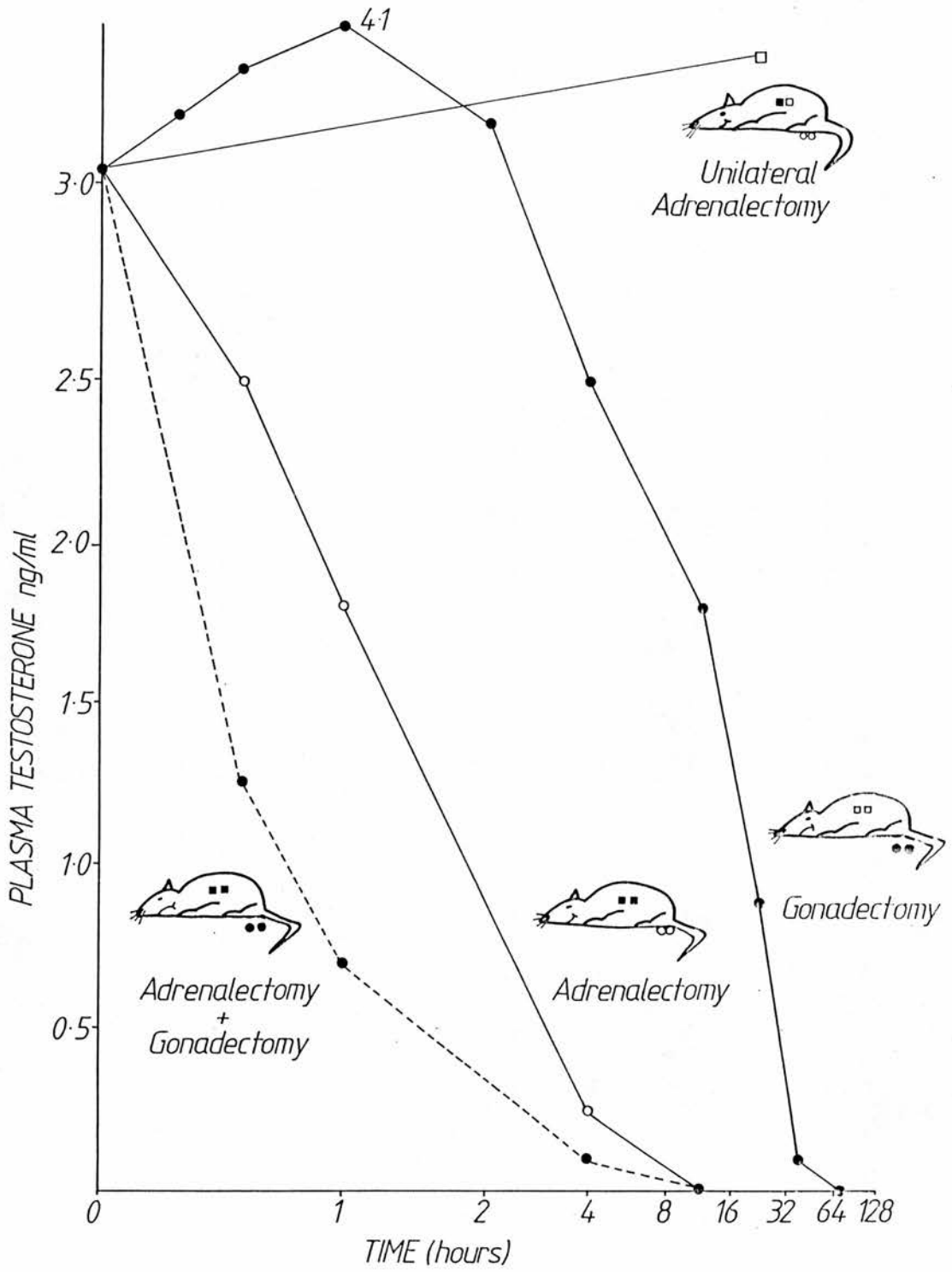


Figure 1.5: The acute effect of adrenalectomy on plasma testosterone levels in the male rat (Kniewald, 1971).

study lacks sham-operated animals as appropriate controls, sampled at time intervals comparable to their adrenalectomised counterparts. It is not possible to determine from this study whether the decline in plasma testosterone levels following surgery is a result of adrenalectomy or the direct result of handling, anaesthesia and surgical stress.

Adrenalectomy performed at the same time as castration delays the post-castration rise in serum gonadotrophin concentrations for up to 12 hours (Schwartz, 1977). These results were initially interpreted as being consistent with a loss of adrenal steroids capable of stimulating gonadotrophin secretion. However, in subsequent experiments it was quite clearly demonstrated that adrenalectomy performed either 12 hours before, or 12 hours after castration also suppressed the rise in gonadotrophins as did sham adrenalectomy (Lorenzen, 1980). Thus adrenalectomy-induced inibition of gonadotrophin secretion cannot be directly mediated by adrenal factors. The administration of the dopamine agonist drug bromocriptine to adrenalectomised and castrated animals, and the administration of ACTH to castrated animals, failed to influence gonadotrophin secretion (Ringstrom, 1984). Thus the adrenalectomy-induced inhibition of gonadotrophin secretion cannot be mediated by prolactin or ACTH. These data are in keeping with a non-specific effect of stress upon gonadotrophin secretion.

ii) In **chronic studies** it has been possible to demonstrate an effect of adrenalectomy upon basal plasma testosterone concen

trations and the acute testosterone response to stress.

a. Adrenalectomy reduces basal testosterone concentrations. Lescoat et al (1982) were able to show that adrenalectomy performed before the onset of puberty profoundly influenced the subsequent maturation of the testis. Bilateral adrenalectomy or sham operation was performed at 25 days of age prior to the estimation of plasma LH and plasma testosterone levels during puberty (30-50 days). Adrenalectomy achieved a significant reduction both in plasma testosterone as well as plasma LH concentrations when compared with sham-operated controls (Fig 1.6). However, the decline in plasma hormone levels in adrenalectomised animals was also associated with reduced weight gain when compared with their sham-operated counterparts. Since adrenalectomised animals did not receive glucocorticoid replacement therapy, chronic glucocorticoid insufficiency may have resulted in a general failure to thrive, which in turn may have accounted for both the reduced weight gain and non-specific suppression of plasma testosterone and gonadotrophin levels. However, by the end of the study the difference in body weight between operated and sham-operated animals was no longer significant.

Nevertheless, it had been previously demonstrated that adrenalectomy performed in adult rats 14 days prior to sampling abolished the normal circadian rhythm of testosterone secretion (Kalra, 1977). Mean $\pm$ SE 0800 and 2200 hours plasma testosterone concentrations were respectively 4.67 $\pm$ 1.14 ng/ml and 1.26 $\pm$ 0.26 ng/ml in sham-operated animals when compared with 1.32 $\pm$ 0.23 and

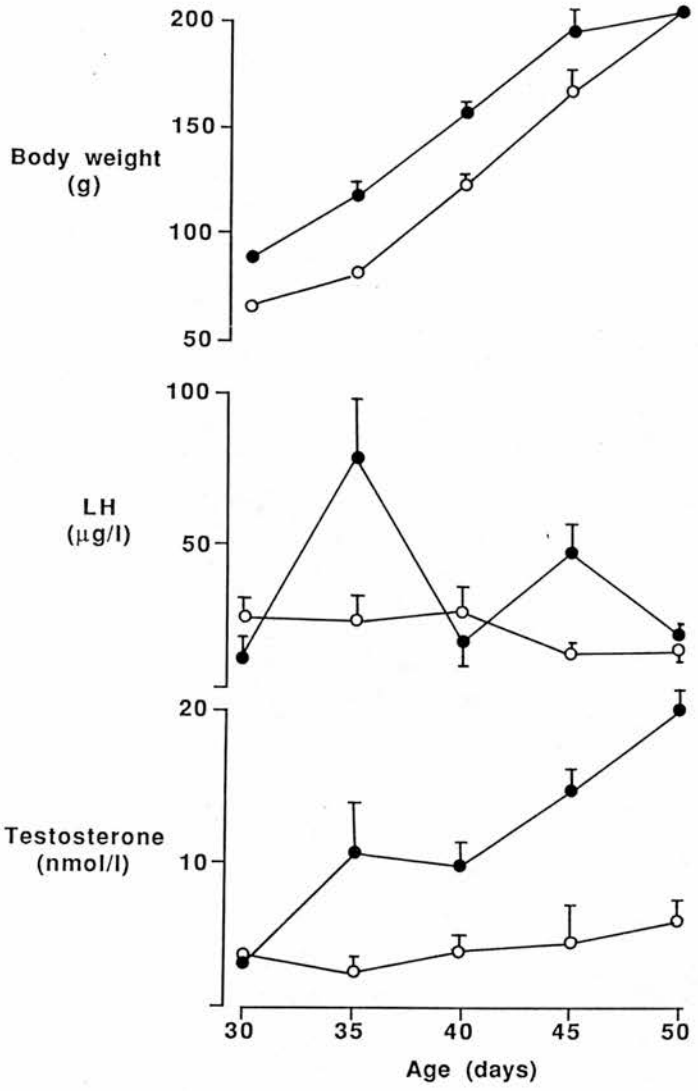


Figure 1.6: Plasma testosterone levels, plasma LH levels and body weight between 30-50 days of age in male rats adrenalectomised (0——0) or sham-adrenalectomised (●——●) at 25 days of age (Lescoat, 1982).

1.49 $\pm$ 0.3 ng/ml in adrenalectomised animals. The reduction in plasma testosterone levels in adrenalectomised animals at this time was not associated with a significant change in either plasma LH or progesterone concentrations when compared with sham-operated controls. These observations strongly suggest that the adrenal gland plays an important role in the circadian regulation of testicular secretion although the circadian variation of serum progesterone does not solely appear to be mediated by the adrenals. However, hypothalamic deafferation abolishes both serum testosterone and progesterone rhythms. Thus, both the mediobasal hypothalamus and the adrenal glands are responsible for the circadian variation in testicular testosterone secretion. Similar findings have been reported by other workers. Adrenalectomy performed in adult rats 10 days before sampling significantly reduced basal plasma testosterone concentrations as well as plasma LH concentrations when compared with sham-operated controls (Lescoat, 1984). However, in contrast to these findings, other workers have been unable to demonstrate any chronic effect of adrenalectomy on plasma testosterone or gonadotrophin levels (McNeilly, 1980). Nevertheless, these data provide persuasive evidence that an adrenal product is capable of stimulating the testis.

b. **Adrenalectomy reduces the acute testosterone response to stress in the rat.** In the acute phase of stress in male rats there is a marked increase in the anterior pituitary secretion of ACTH, prolactin and gonadotrophin, increased secretion of corticosterone

and progesterone from the adrenal gland and increased secretion of testosterone from the testis. Since testosterone levels in the blood fall to undetectable concentrations following castration, the direct adrenal contribution to plasma testosterone concentrations is negligible (Kniewald, 1971; Kalra, 1977). Yet, adrenalectomy significantly reduces the rise in plasma testosterone concentrations that occurs during the acute phase of stress, suggesting that the acute testosterone response is mediated indirectly by the adrenal (Lescoat, 1984).

1.4.2: Adrenal ⟶ gonad interactions in vivo: the effect of ACTH and adrenal corticosteroids on gonadal function in the male rat

It has been well established that the administration of ACTH to male rats inhibits testicular function (Baker, 1950; Asling, 1951; Saez, 1977;), and that this inhibitory function requires the presence of the adrenal gland to exert its effect (Mann, 1982). Administration of ACTH to intact male rats increases serum concentrations of corticosterone and progesterone and decreases serum concentrations of LH, FSH and testosterone (Vreeburg, 1984). Depression of testicular function by ACTH is presumably mediated by the adrenal secretion of corticosteroids.

i) Glucocorticoids suppress the testicular secretion of testosterone acting either directly upon the testis (Desjardins, 1971; Saez, 1977; Bambino, 1981; Welsh, 1982) or indirectly through anterior pituitary gonadotrophs (Smith, 1971; Baldwin, 1974; Baldwin, 1979). Administration of pharmacological doses of

dexamethasone (600 µg daily for 3 days) to prepubertal rats markedly inhibits hCG-stimulated testicular steroidogenesis and appears to act by reducing the number of hCG-binding sites (Saez, 1977). These findings have been confirmed in adult rats employing lower doses of dexamethasone (10-100 µg daily for 5 days) (Bambino, 1981). The administration of corticosterone in supraphysiological doses to castrated animals increases the sensitivity of the negative-feedback response to testosterone (Vreeburg, 1984). It was also demonstrated that the administration of supraphysiological doses of cortisol (100 mg pellets by subcutaneous implantation) over a 3-day period significantly suppresses plasma LH concentrations in animals 12 hours post-castration, confirming that glucocorticoids may exert an inhibitory effect over gonadotrophin secretion. However, other studies have failed to demonstrate any effect of ACTH or glucocorticoid in either intact or castrated rats (Verjans, 1976). The reasons for these discrepancies are not immediately clear.

ii) Progesterone levels fluctuate in the blood of male rats with daily periodicity, and the rise in plasma progesterone concentrations is casually related to a biphasic secretion of testosterone. Progesterone administration to either intact or adrenalectomised male rats is effective in raising both plasma progesterone and testosterone levels without affecting plasma gonadotrophins and its administration to hypophysectomised male rats achieves similar results (Kalra, 1980). This confirms previous observations that the administration of progesterone to

intact or castrated male rats has little influence upon the anterior pituitary secretion of gonadotrophins (Kingsley, 1973; Brown-Grant, 1974; Aiyer 1976; Fink, 1976). Progesterone stimulates testicular steroidogenesis (Kalra, 1980) and this is presumably mediated by direct means rather than indirectly through the stimulation of the anterior pituitary secretion of LH.

1.4.3: Adrenal ➔ gonad interactions in vitro: the effect of the adrenal secretion of corticosteroids on testicular steroidogenesis in the rat

Other studies have provided in vitro evidence that the adrenal gland is capable of influencing the testis. Using whole organs it has been possible to demonstrate that the administration of ACTH to fetal testes only increases testicular steroidogenesis when the testes are incubated in the presence of fetal adrenal tissue and not when the two tissues are separate (Warren, 1984b). This strongly suggests that the fetal adrenal gland may play an important role in the regulation of testosterone production by the fetal testis. Furthermore, the fetal adrenal alone fails to secrete significant amounts of testosterone in response to ACTH.

The novel technique of electrofusion has also been used to demonstrate adrenal ➔ gonad interactions (Podesta, 1984). This technique produces a hybrid line of adrenal-Leydig cells that are encompassed by one cell membrane. This hybrid cell secretes corticosterone in response to the administration of LH and testosterone in response to ACTH, whereas isolated adrenal and Leydig

cells alone fail to respond in this way. Moreover, these authors were unable to demonstrate cooperation between adrenal and Leydig cells in the biosynthesis of testosterone when incubated in the presence of ACTH in static incubations.

In vitro methods have also been used to demonstrate the ability of isolated Leydig cells (Hall, 1969) and Sertoli cells (Hall, 1969; Tcholakian, 1978) to metabolise progesterone to testosterone. Intriguingly, the ability of the testis to utilise progesterone is limited to certain periods of testicular maturation (Steinberger, 1968). The addition of physiological concentrations of progesterone to fetal testes stimulates testosterone production (Habert, 1986). Testicular tissue derived from newborn rats converts approximately 25-60% of progesterone to androgens but this ability is lost (less than 1%) as the testes mature over the period 10-40 days of age, rising again to 40% in the post-pubertal animal (Steinberger, 1968).

The mechanism of steroid entry into cells has recently come under scrutiny (Ballard, 1979). It has been generally assumed that glucocorticoids enter cells by passive diffusion by virtue of their small molecular size and lipophilic nature. Certainly the diffusion of naturally occurring glucocorticoids as well as synthetic glucocorticoids is proportional to the extracellular concentration of steroid (Samuels, 1970; Mayer, 1976; Plagemann, 1976). Other authors have argued for specialised transport systems and propose that the cell membrane contains a component that binds glucocorticoid and acts in a transport capacity (Brinkmann, 1972;

Milgrom, 1973; Harrison, 1974; Harrison, 1975; Harrison 1976; Rao, 1976; Harrison, 1977; Rao, 1977). Binding of glucocorticoid to the cell membrane was achieved by two classes of receptor and inhibited by prior exposure of the cell membrane to enzymes; uptake of glucocorticoid into cells was under certain circumstances found to be temperature-dependent. However, more recent studies have disputed this concept and have demonstrated that velocity of passage of a wide range of naturally occurring steroids through the plasma membrane is extremely rapid (of the order of 10^4 cm sec^{-1}) and occurs by simple diffusion (Giorgi, 1981). This applies to free steroids and not to their conjugated counterparts. Furthermore, the velocity of passage of steroid through the membrane is closely related to the partition coefficient of that steroid in n-octanol and water. Therefore it is likely that the non-polar steroids such as progesterone will diffuse across membranes with greater ease than polar steroids.

1.5: Conclusions

The notion that the adrenal gland may influence the testicular secretion of testosterone in the rat is persuasive and based upon the following hypothesis:

i) **Acute stress** stimulates the hypothalamic-pituitary-adrenal axis stimulating the adrenal secretion of corticosterone and progesterone. The adrenal secretion of progesterone acts as a direct positive signal whereas corticosterone acts as an indirect negative signal to teststicular steroidogenesis.

ii) **Chronic stress** exerts a non-specific inhibitory effect on the hypothalamic-pituitary-testicular axis by suppressing the anterior pituitary secretion of gonadotrophin.

The adrenal gland may therefore provide important external signals to the internal rhythms of the hypothalamic-pituitary-testicular axis. These signals may be of importance in their effect upon the reproductive potential of the animal and may contribute to the stress response of the male rat. The hormonal response to mating is similar to that of acute stress resulting in increased secretion of LH, prolactin and testosterone (Kamel, 1978). During the acute phase of stress the adrenal contribution of progesterone stimulates the testicular secretion of testosterone, whereas the adrenal secretion of corticosterone suppresses the testicular secretion of this hormone. The interaction of adrenal corticosteroids with the hypothalamo-pituitary-testicular axis is therefore complex and the resultant effect on testicular steroidogenesis may depend upon the balance of these two signals. In the chronic phase of stress the overriding response is suppression of the testicular secretion of testosterone and this is presumably mediated through inihibition of GnRH and the anterior pituitary secretion of LH.

The purpose of this thesis is to demonstrate that adrenal corticosteroids influence the testicular secretion of testosterone both *in vitro* and in *in vivo*.

Chapter 2

MATERIALS AND METHODS

2.1: Animals and in vivo experimental techniques

All the animals used for these studies were male Wistar rats bred from the colony at the Animal Unit, Western General Hospital, Edinburgh. Rats were housed under conventional conditions at a temperature of 19-21°C, with a light:dark cycle comprising 12 h light and 12 h dark and fed ad libitum on rat chow (CRM(X) diet, Labsure UK) and water. In order to prevent differences that may relate to age, pubertal status and weight, rats were usually bred specifically for each experiment, thus reducing any variation between the groups. In the course of these studies the following techniques were employed for in vivo experiments.

2.1.1: Anaesthesia

General anaesthesia was administered by an open method using a mixture of Halothane (Fluothane, ICI UK) and 100% oxygen (British Oxygen Company, UK) at a rate of 2 litres per minute via a vaporiser (Fluotec Mk 3, Cyprane UK). Induction of anaesthesia was usually achieved by placing the rat in a 'dessicator jar' with a continuous flow of 4% halothane in oxygen. As soon as the rat was unconscious, it was placed on the operating table with its tail towards the operator and anaesthesia maintained through a face mask (plastic filter funnel) employing 1-3% halothane in oxygen.

2.1.2: Adrenalectomy

Adrenalectomy (Waynford, 1980a) was performed by placing the anaesthetised rat on its ventral surface with its tail towards the

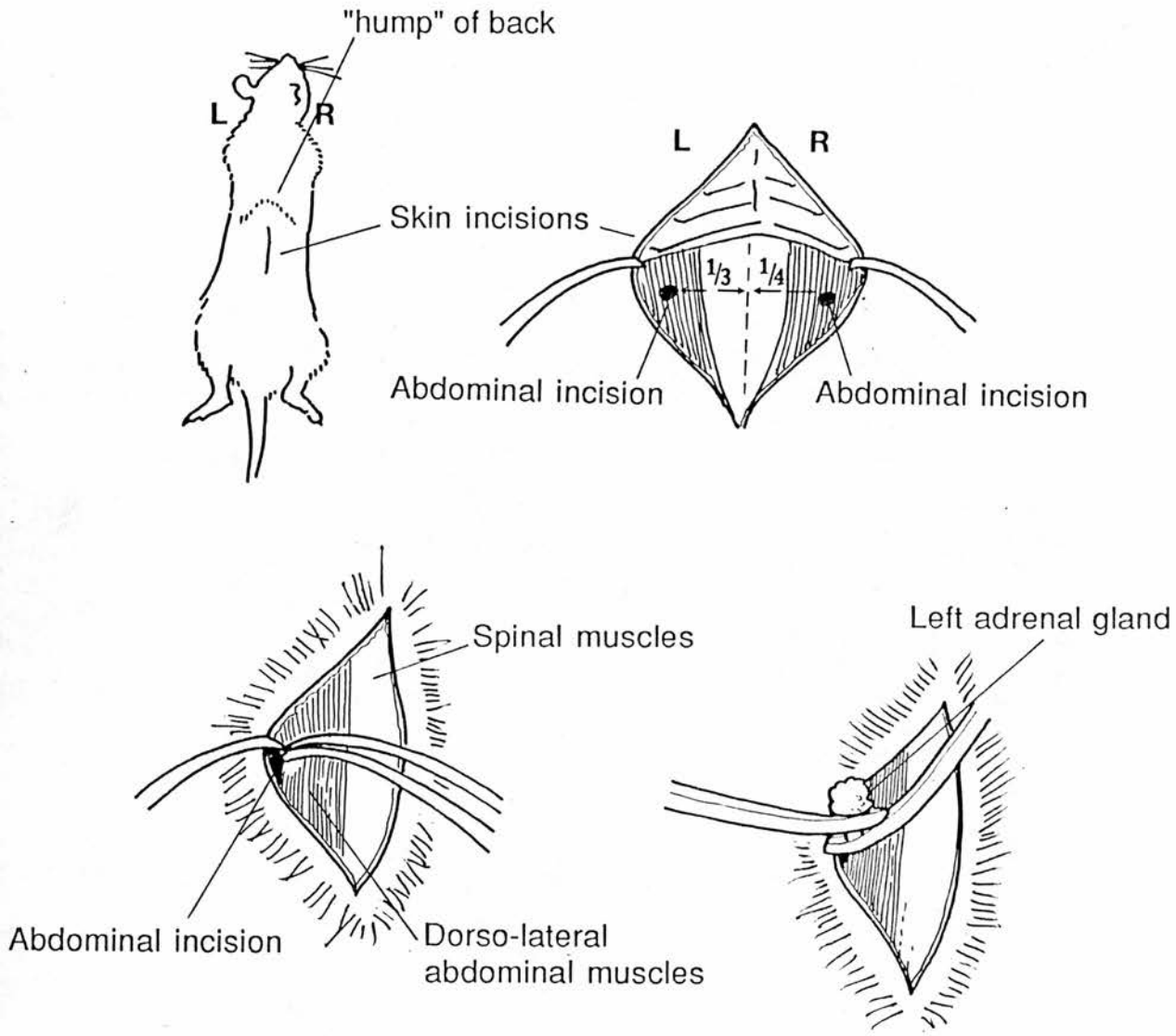


Figure 2.1: Technique of bilateral adrenalectomy in the rat - see text for details.

operator, the skin cleansed with a solution of 0.5% chlorhexidine in alcohol 70% and the operation performed under aseptic conditions. The hump on the back of the animal was identified and a 2-3 cm midline incision was made with a scalpel. The connective tissue was cleared away by blunt dissection using curved scissors to expose the posterior abdominal wall. An incision was made by means of curved scissors into the abdominal muscle on the left side just posterior to the last rib and approximately one third down the side of the animal on the left. Access was gained to the peritoneal cavity which was gently explored using two pairs of curved forceps and the **left adrenal** identified - it was usually found close to the spine, between spleen and kidney. Once the adrenal gland had been found the pedicle to the gland was grasped with the forceps in the left hand and the right-hand pair was inserted deeply to grasp the pedicle below the first pair. The gland itself was not touched in case it ruptured and reimplanted itself in the abdominal cavity. The adrenal gland was removed by severing its pedicle by pulling the forceps away from each other. The approach to the **right adrenal** is similar, with an incision one quarter down the side of the animal on the right. The adrenal was usually found between the kidney and the liver and the process of removal of the right adrenal is similar to that of the left (Fig 2.1). No haemostasis was required and providing access to the peritoneum was through a small incision no suture was necessary. However, the midline skin incision was closed with two 11 mm skin clips (Michel, Germany) using the appropriate clip applicator

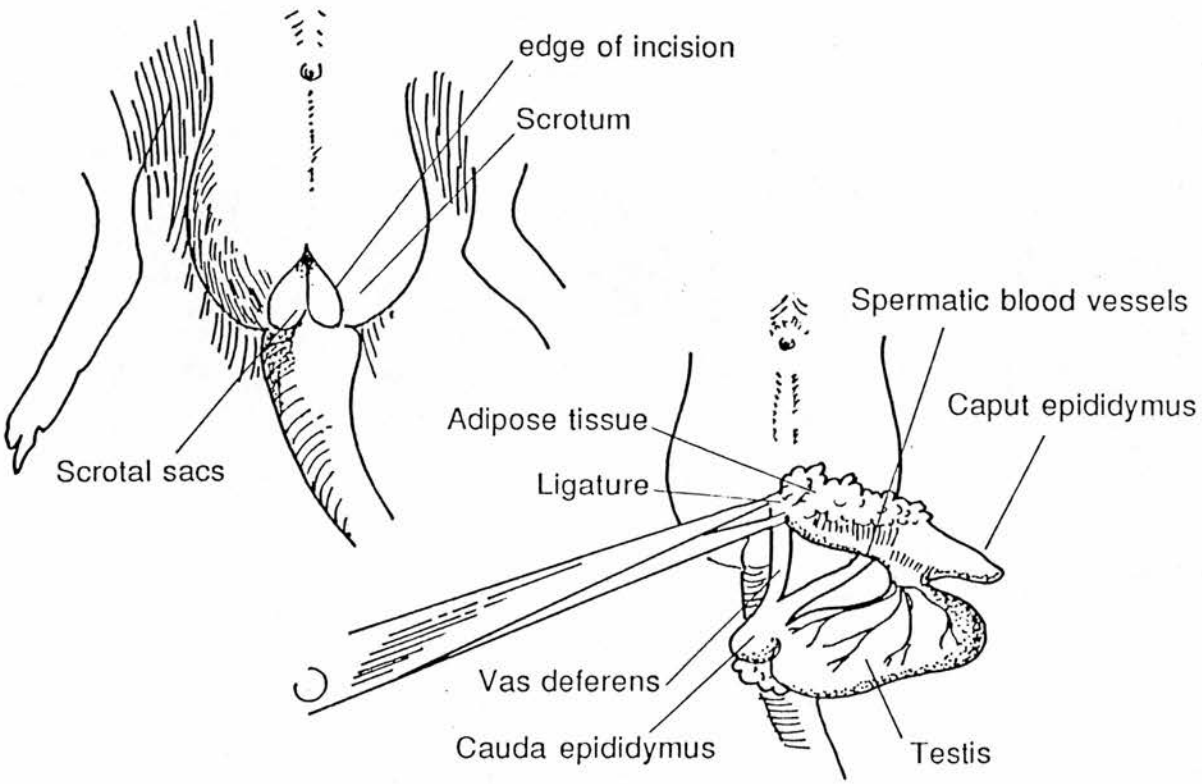


Figure 2.2: Technique of orchidectomy in the rat - see text for details.

(Roboz RS-9294, Germany). Postoperative corticosteroid replacement was not necessary providing the animals were given ad libitum physiological saline (0.9% w/v) to drink. The success of total bilateral adrenalectomy was subsequently confirmed by the finding of undetectable plasma corticosterone concentrations and any animal with detectable plasma cortcosterone levels was excluded from data analysis.

2.1.3: Sham adrenalectomy

Sham adrenalectomy was performed under general anaesthesia by gaining access to the peritoneal cavity on both sides, in order to identify both **left** and **right adrenal glands,** without removal. The peritoneal cavity and skin incisions were then closed as for bilateral adrenalectomy.

2.1.4: Orchidectomy

Orchidectomy (Waynford, 1980b) was performed by placing the anaesthetised animal on its back with its tail towards the operator. The operation was performed under aseptic conditions and the scrotum cleansed with a solution of 0.5% chlorhexidine in alcohol 70%. A 1 cm median incision was made in the tip of the scrotum with a pair of scissors and the connective tissue cleared with blunt dissection to reveal the testes in their muscular sacs. Pressure was often required to bring the testes down. A small incision was then made at the tip of the muscular sac with a pair of scissors sufficient to allow the passage of the cauda

epididymis, caput epididymis, vas deferens and spermatic blood vessels. A silk ligature (Mersilk, Ethicon, UK) was placed around the vas deferens and spermatic blood vessels and divided distal to the ligature permitting removal of the testis. The remaining piece of vas was then pushed back into the sac and the incision closed with a silk suture to prevent herniation. The other testis was removed in the same way (Fig 2.2). The scrotal incision was then closed with two 11 mm skin clips.

2.1.5: Administration of agents

i) ACTH: Groups of rats were injected either with 0.5 ml sterile physiological sodium chloride (0.9% w/v) (controls) or ACTH 1-24 (Tetracosactrin, CIBA-Geigy Laboratories, Switzerland) over the dose range 0.01-10 µg in 0.5 ml sterile physiological sodium chloride (0.9% w/v) by intraperitoneal route.

ii) Progesterone: Groups of rats were injected either with 0.5 ml sterile physiological sodium chloride (0.9% w/v) (controls) or 100 µg progesterone (Sigma Chemical Company, USA) in 0.5 ml sterile physiological saline (0.9% w/v) by subcutaneous injection.

iii) Dexamethasone: Groups of rats were administered either with 0.5 ml tap water (controls) or with dexamethasone (Merck Sharpe and Dohme, UK) 100 µg in 0.5 ml tap water using an oral intragastric blunt-ended dosing needle. Dexamethasone was administered at 0900, 1700 and 2300 h on the day before the study day and again at 0900 h on the day of the study.

2.1.6: Blood sampling

Blood was collected from experimental animals by decapitation into tubes containing lithium heparin (LH10, Brunswick, UK) and then separated by centrifugation (MSE Coolspin, Fisons UK) at 2000 g for 15 minutes at 4°C and the plasma layer removed and stored (approximately 2.5 ml) at -20°C until analysis for appropriate steroid and LH concentrations. Great care was taken to avoid stress in the experimental animals which were housed in a separate room from that in which they were sacrificed. 'Companion' animals were introduced at the time of experimentation in an attempt to reduce stress that may result from isolation of the remaining rats during sacrifice. Each experimental cage usually contained up to eight experimental animals plus two 'companions'. Sacrifice was performed in an adjacent room and the time from removing the animal from its cage to decapitation was less than 20 seconds. Animals were sacrificed on a rota basis, alternating with each experimental group. All procedures were approved by the Home Office Animals Inspectorate and licensed under the Cruelty to Animals Act 1876.

2.1.7: In vivo steroid hormone and LH assays

Steroid hormone analysis in plasma was performed by direct (unextracted) and indirect (extracted) radioimmunoassay for progesterone (Ratcliffe, 1982), testosterone (Corker, 1978), corticosterone (Al-Dujaili, 1981b) and LH (Fraser, 1977), all employing ^{125}I-radioligands. Plasma samples for estimation of progesterone and LH concentrations were assayed neat, whereas

plasma samples for estimation of testosterone concentrations were assayed after dilution (1:5) following extraction, and samples for corticosterone estimation were assayed after dilution (1:10).

2.1.7.1: Plasma progesterone assay

For direct assay, 50 µl of sample or standard (over the range 0.31 to 40 ng/ml in charcoal-stripped rat plasma) was added to 100 µl antibody (R31/7 raised in rabbits against 11α-hemisuccinyl-progesterone) in EDTA-phosphate assay-buffer (initial dilution 1:4,500; final dilution 1:25,000) and 400 µl of tracer (^{125}I-progesterone-11α-glucuronyl tyramine, 10,000 cpm) in assay-buffer, containing danazol (400 ng in 400 µl or per tube) (17α-pregna-2,4-dien-20-yon(2,3-d)isoxazol, Winthrop Laboratories, UK) and incubated in borosilicate tubes (10 x 75 mm, Corning, USA) at room temperature for 4 hours prior to the addition of second antibody. Borosilicate tubes were used in preference to plastic because of the nature of progesterone and its possible binding to plastic. The purpose of the addition of the danazol was to displace progesterone from binding to serum proteins, reducing that bound from 35-40% to less than 4% with the amount of danazol used (McGinley, 1979). Precipitation of tracer bound to antibody was achieved with second antibody, consisting of 100 µl donkey anti-rabbit antiserum (initial dilution 1:20; final dilution 1:150) and 100 µl normal rabbit serum (initial dilution 1:200; final dilution 1:1500), provided by the Scottish Antibody Production Unit (Carluke, Scotland). Following addition of the second antibody,

tubes were incubated overnight at 4°C and then centrifuged (MSE, Coolspin, Fisons UK) at 2000 g for 30 minutes at 4°C, the supernatant decanted and the precipitate (bound) counted in a gamma-counter (LKB-Wallac, UK). EDTA-phosphate assay-buffer (pH 7.4) consisted of:

Na_2HPO_4 (8.52 g/l) (AnalaR, BDH, UK)

Na_2EDTA (4.7 g/l) (AnalaR, BDH, UK)

BSA (0.25% w/v, fraction V, Miles, UK)

The final pH was adjusted to 7.4. The antibody was a gift from Dr J E T Corrie, Department of Medicine, Western General Hospital, Edinburgh and the tracer provided by the Department of Clinical Biochemistry, Royal Infirmary, Glasgow.

Profiles for intra- and inter-assay precision are illustrated in figure 2.3. Precision is calculated from five consecutive standard curves as the coefficient of variation of the dose estimate, derived from the standard deviation of dose estimate divided by the dose (Ekins, 1983), and expressed as (CV%). Over the working range 0.5 to 10 ng/ml intra- and inter-assay precision are respectively 6% and 15% at 0.5 ng/ml and 10% and 7.5% at 10 ng/ml. The working range was usually determined by the limits 90-10% B/B_o (see figure 2.4 for a typical standard curve) as well as the precision profile.

Double dilution of rat plasma produced dilution curves parallel to the standard curve (Fig 2.4) and cross-reactivities to possible interfering steroids are in close agreement with those previously published. There is no significant cross-reactivity

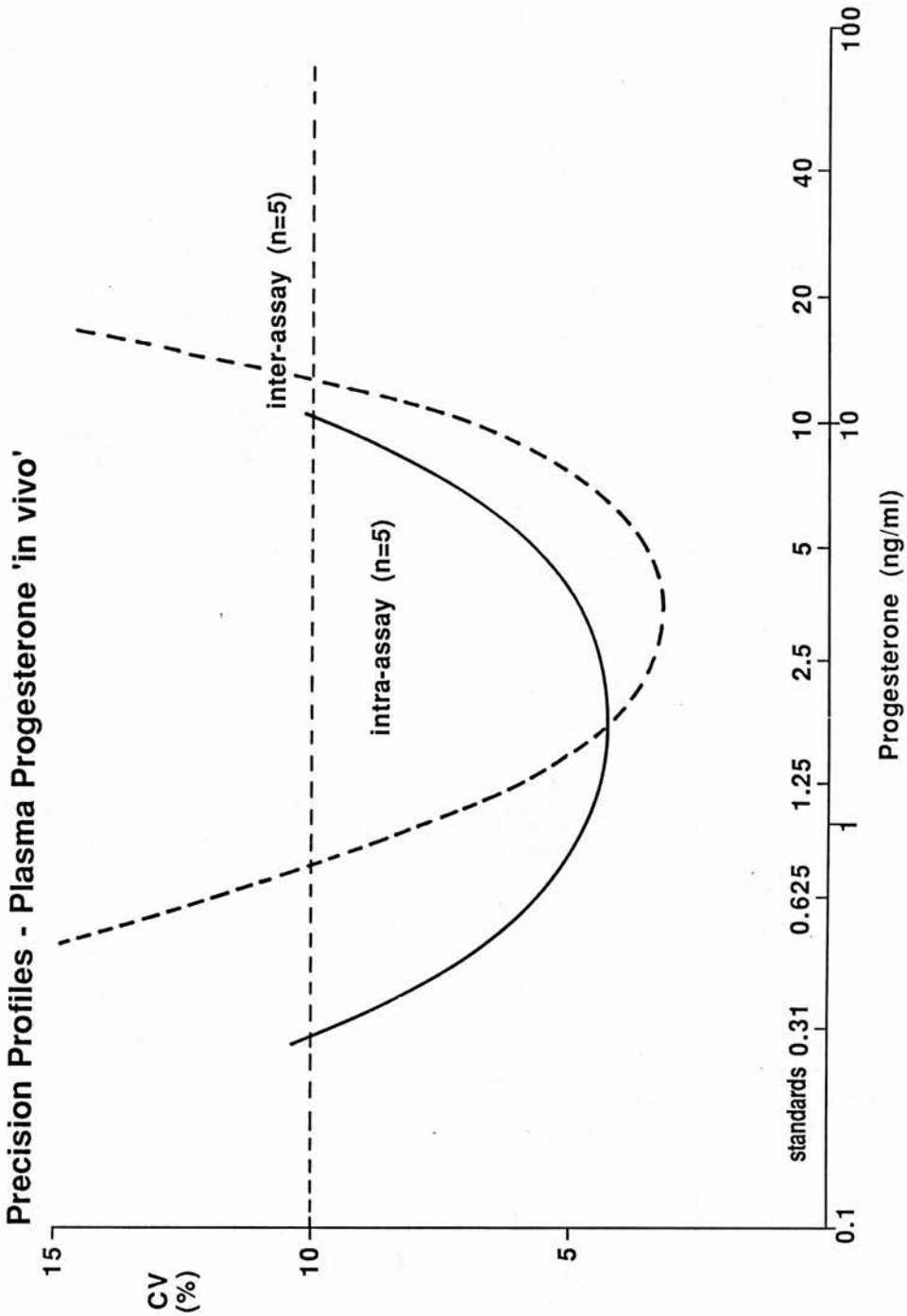


Figure 2.3: Precision profiles for plasma progesterone assay.

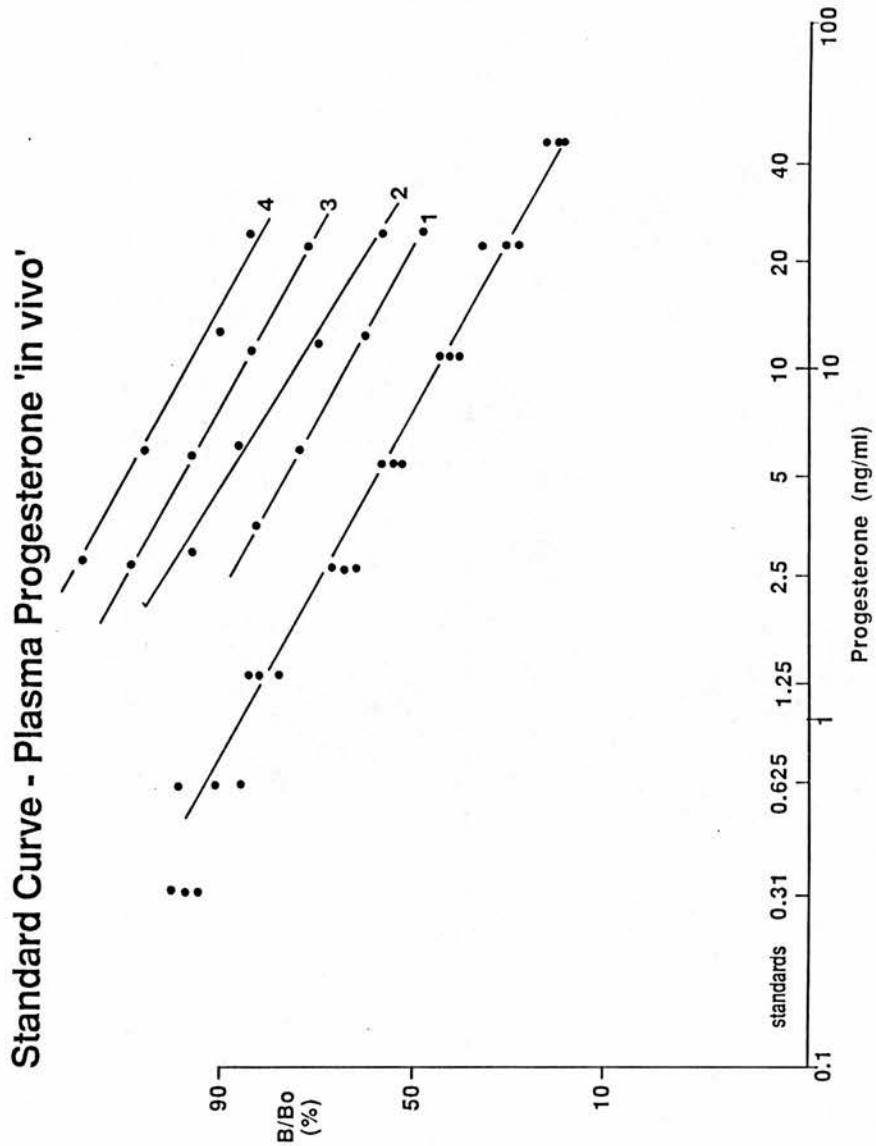


Figure 2.4: A typical standard curve for assay of plasma progesterone with dilutional studies.

with the synthetic glucocorticoid dexamethasone. This assay compares favourably with other progesterone assays for rat plasma (Kalra, 1977; Lorenzen, 1980).

2.1.7.2: Plasma testosterone assay

For indirect assay testosterone was first extracted from rat plasma samples by incubating 100 µl of sample with 2 ml diethyl ether (Anaesthetic ether, BP, May and Baker, UK), shaking at room temperature for 5 minutes in a glass tube (Sterilin, UK). The aqueous layer was frozen with solid carbon dioxide and the organic layer decanted into borosilicate tubes (Corning Laboratories, USA) and evaporated to dryness under a nitrogen stream at room temperature. The extracted plasma was reconstituted in 0.5 ml diethyl ether and evaporated again to dryness before reconstituting with 500 µl (1:5 dilution) of 0.1 molar phosphate assay buffer (pH 7.4) with gelatin (0.1% w/v) and vortexed (Fisons, UK) for 20 seconds. 0.1 molar phosphate assay buffer (pH 7.4) consisted of:

NaCl (9 g/l)

Na_2HPO_4.anhydrous (8.6 g/l)

NaH_2PO_4. anhydrous (4.68 g/l)

All reagents were obtained from AnalaR, British Drug Houses, UK. Gelatin (1 g/l) (G-2500 Sigma Chemicals, USA) was dissolved in warmed buffer and the final pH adjusted to 7.4 with sodium hydroxide. Efficiency of extraction was assessed for each extraction by incubating 10 µl of ^3H-testosterone (Amersham, UK, to give approximately 10,000 counts per minute; specific activity 3.4

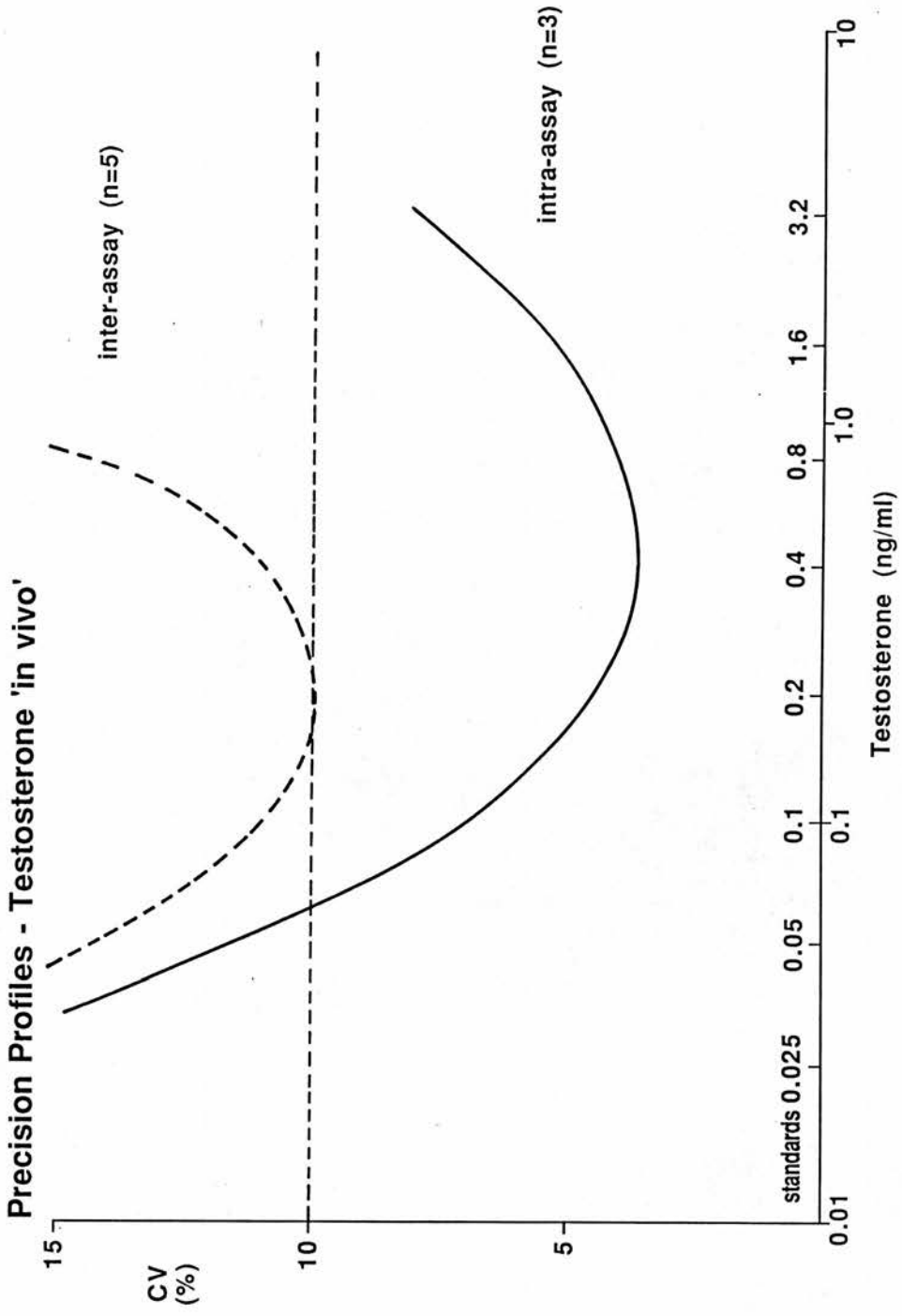


Figure 2.5: Precision profiles for plasma testosterone assay.

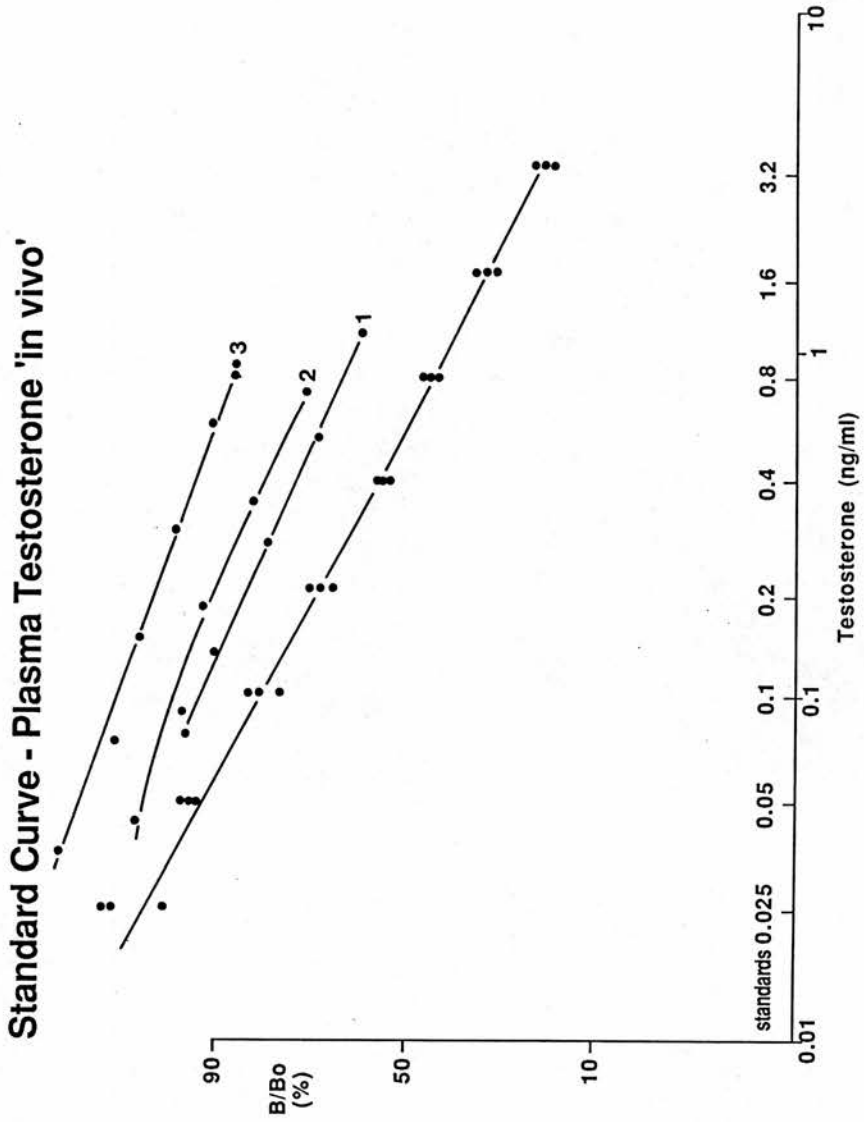


Figure 2.6: A typical standard curve for plasma testosterone with dilutional studies.

TBq/nmol) in 3 ml charcoal-stripped rat plasma for at least two hours prior to extraction in parallel with samples for assay. Average recovery was 93%.

For assay, 100 µl reconstituted (extracted) sample or standard (over the dose range 0.025 ng/ml to 3.2 ng/ml in 0.1 molar phosphate/gelatin buffer containing charcoal-stripped rat plasma 10% w/v) was incubated overnight at 4°C with 100 µl antibody (E01, raised in the goat against testosterone-3-(-O-carboxymethyl)-oxime-BSA, initial dilution 1:10,000; final dilution 1:30,000) and 100 µl tracer (testosterone-3-(-O-carboxymethyl)-oxime-^{125}I-histamine, 3000 counts per minute), both in 0.1 molar phosphate-gelatin buffer. Assays were incubated in plastic LP3 (Luckhams Ltd, UK) tubes overnight at 4°C prior to separation by the addition of 1 ml 0.5% (w/v) dextran-coated charcoal in 0.1 molar phosphate buffer and centrifuged (MSE Coolspin, Fisons, UK) at 2000 g for 15 minutes at 4°C. The supernatant was aspirated, discarded and the remaining pellet, unbound tracer (free) counted in a gamma counter (LKB-Wallac, UK). The values were corrected for percentage recovery and dilution. The antibody is a gift from the M R C Reproductive Biology Unit, Chalmers Street, Edinburgh and tracer was prepared by coupling testosterone-3-(-O-carboxymethyl)-oxime to ^{125}I-histamine as described under <u>in vitro</u> methods (see section 2.2.5.6).

Profiles of intra- and inter-assay precision (CV%) are illustrated in figure 2.5. Over the working range 0.1 to 1.6 ng/ml the intra- and inter-assay precisions are respectively 6.8% and 10.9% at 0.1 ng/ml and 5.5% and 13.0% at 1.6 ng/ml. The working range

was usually determined by the limits 90-10% B/B_0 (see figure 2.6 for a typical standard curve) as well as the precision profile. Extracted samples when diluted, demonstrated parallelism to the standard curve (Fig 2.6) and cross-reactivities to possible interfering steroids are similar to those previously reported (Bartlett, 1985). There is no significant cross-reactivity with the glucocorticoid dexamethasone. This assay compares favourably with other testosterone assays for rat plasma (Lescoat, 1982; Wheeler, 1983; Stahl, 1984; Bartlett, 1985).

2.1.7.3: Plasma corticosterone assay

For direct assay 50 µl sample (diluted in Krebs-Ringer buffer (1:10) containing BSA 0.2% (w/v) (fraction V, Miles Reagents, UK) or standard (over the range 0.5 to 64 ng/ml in Krebs-Ringer buffer containing BSA 0.2% (w/v) and charcoal-stripped rat plasma 10% (v/v)) was added to 200 µl of antibody (R1/B3 raised in rabbits against corticosterone-3-(-O-carboxymethyl)-oxime-BSA) (initial dilution 1:20,000; final dilution 1:45,000) and 200 µl tracer (corticosterone-3-(-O-carboxymethyl)-oxime-(I^{125})-histamine, 3000 counts per minute, both assayed in phosphate/citrate bufffer pH 3.0 to displace corticosterone from its binding protein (Al-Dujaili, 1981b). Assay buffer (pH3.0) consisted of:

Na_2HPO_4 (14.2 g/l)

Citric acid monohydrate (21 g/l)

BSA (0.1% w/v) (fraction V, Miles Reagents, UK).

Assay samples were incubated overnight at 4°C and separated by the

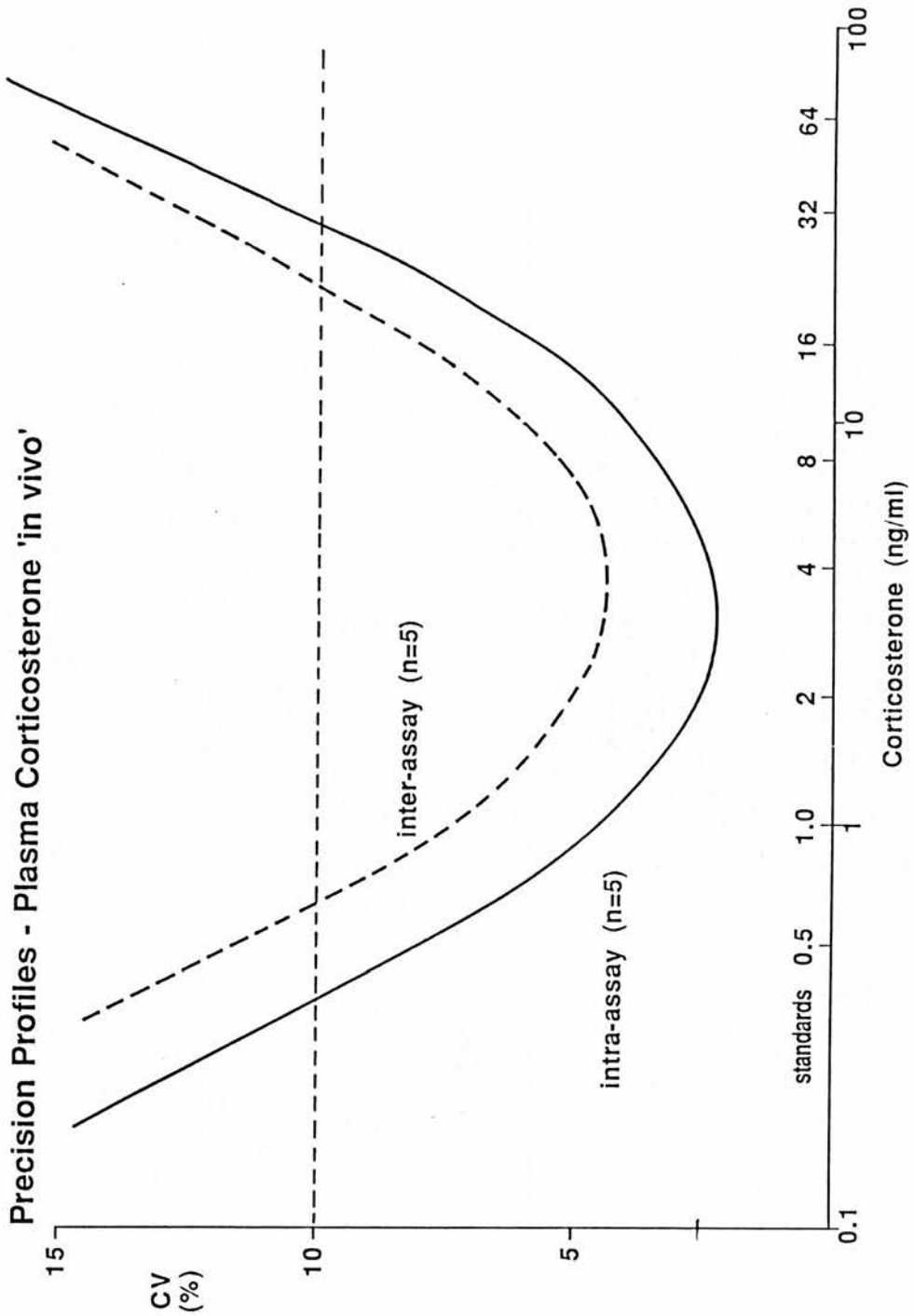


Figure 2.7: Precision profiles for plasma corticosterone assay.

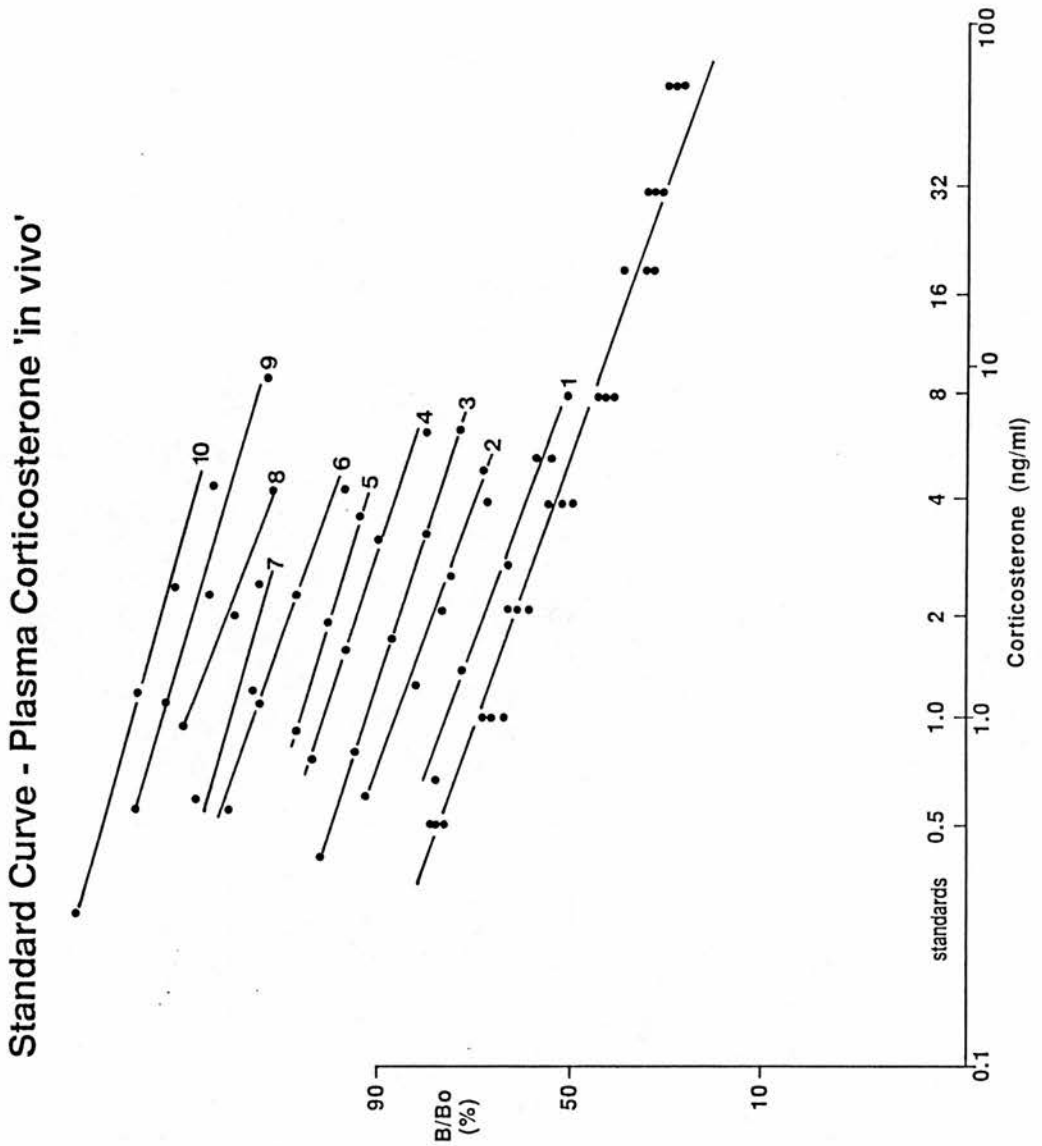


Figure 2.8: A typical standard curve for plasma corticosterone with dilutional studies.

addition of 1 ml dextran-coated charcoal and centrifuged (MSE Coolspin, Fisons UK) at 2000 g at 4°C for 15 minutes. The supernatant was aspirated, discarded and the remaining pellet, unbound (free) tracer, is counted in a gamma counter (LKB-Wallac). The antibody is a gift from Dr B C Williams, Department of Medicine, Western General Hospital, Edinburgh and tracer was prepared by coupling corticosterone-3-(-O-carboxylmethyl)-oxime to ^{125}I-histamine as described under in vitro methods (see section 2.2.5.6).

Profiles for intra- and inter-assay precision are illustrated in figure 2.7. Over the working range 0.5 to 16 ng/ml the intra- and inter-assay precisions are respectively 8% and 11.1% at 0.5 ng/ml and 6% and 7.5% at 16 ng/ml. The working range was usually determined by the limits 90-10% B/B_0 (see figure 2.8 for a typical standard curve) as well as the precision profile.

Double dilution of rat plasma produced dilutional curves parallel to the standard curve (Fig 2.8) and cross-reactivities to possible interfering steroids are in close agreement with those previously published. There is no significant cross-reactivity with the synthetic glucocorticoid dexamethasone. This compares favourably with other corticosterone assays for rat plasma (Lescoat, 1984).

2.1.7.4: Plasma LH assay

Plasma levels of LH were measured by direct radioimmunoassay as described by Fraser and Sandow (1977) and I am grateful to Dr R

Sharpe, M R C Reproductive Biology Unit, Chalmers Street, Edinburgh for supplying the reagents.

2.1.7.5: Preparation of charcoal-stripped rat plasma

For preparation of standards containing known quantities of appropriate hormones for radioimmunoassay, it is necessary to remove all endogenous steroids by charcoal-stripping. Activated charcoal (Sigma Chemicals, USA) was washed three times with 0.1 molar phosphate buffer (pH 7.4 containing BSA (0.1% w/v)) in a ratio of 0.3 g charcoal to 10 ml buffer. The charcoal/buffer slurry was centrifuged (MSE Coolspin, Fisons, UK) between washes at 300 g at 4°C and the supernatant containing fine charcoal discarded on each occasion. Rat plasma was clock-stirred overnight with the washed charcoal in a ratio of 1 g (dry weight) charcoal to 10 ml rat plasma. The plasma/charcoal slurry was then centrifuged at 2000 g for 1 hour and the supernatant decanted as charcoal-stripped rat plasma. The efficiency of stripping was assessed by incubating 3 ml of the original rat plasma with 50 µl ^{3}H-corticosterone for 2 hours at room temperature. The 'spiked' sample was treated in parallel in the same way as the plasma intended for use as standards. On average, 95% of ^{3}H-corticosterone was removed by this process. Plasma samples were then prepared as standards by the addition of appropriate concentrations of appropriate hormone, aliquoted and stored at -20°C until use.

2.1.8: Statistics:

Statistical differences between groups were analysed by Wilcoxon test for paired and unpaired data as appropriate, using a BBC microcomputer (software, Dr C S Hetherington, Department of Clinical Chemistry, University of Newcastle-upon-Tyne).

2.2: Materials and methods of in vitro techniques

Lowry and McMartin first described a superfusion system to investigate steroidogenesis by isolated rat adrenal cells in vitro in 1974. Cells were obtained by enzymatic digestion and suspended on a polyacrylamide matrix using a 2 ml syringe as a superfusion chamber. One or two superfusion chambers were used for each experiment. This system has been adapted to a multicolumn isolated cell superfusion system and used to investigate the interaction between isolated rat adrenal and testicular cells in the secretion of sex steroids. This system has the advantage of comparing steroidogenesis from 10 parallel columns within each experiment.

2.2.1: Preparation of isolated whole adrenal cells

Ten adult male Wistar rats (250 g) were killed by carbon dioxide narcosis and whole adrenals identified and removed. The adrenal glands were cleaned of adhering fat using fine forceps and a pair of fine scissors, then minced and digested. Digest took place in a 100 ml pot (Sterilin, UK) containing a 10 ml solution of collagenase (Worthington Diagnostic Systems, Flow Laboratories, UK) at a concentration of 2 mg/ml in Krebs-Ringer buffer containing bovine serum albumen (1% w/v) (BSA, fraction V, reagent grade,

Figure 2.9: Photograph of the superfusion system.

Miles Laboratories, UK) and glucose (0.2% w/v) (AnalaR, BDH, UK) for 60 minutes in a shaking water bath (Grant Instruments, UK) at 37°C and 120 cycles per minute (Tait, 1972). Cells were mechanically dispersed with a 5 ml pipette at 30 and 60 minutes and isolated cells separated from debris by filtration through a 100 μ nylon gauze (Henry Simon Ltd, UK). Isolated adrenal cells were then centrifuged at 300 g for 15 minutes at 10°C (MSE Coolspin, Fisons, UK). The supernatant was discarded and the cell pellet resuspended in Krebs-Ringer buffer and the cells counted and adjusted to 1 x 10^6 cells suspended in 0.5 ml so that 1 x 10^6 could be added to each superfusion chamber according to the experimental protocol. This preparation is therefore unpurified containing adrenocortical cells of the zona glomerulosa, fasciculata and reticularis as well as from the adrenal medulla.

2.2.2: Preparation of isolated testicular cells

Ten male Wistar rats (250 g) were killed by carbon dioxide narcosis and whole testes removed with the minimum of trauma. Testicular cells were prepared by stripping the capsule from each whole testis with the absolute minimum of mechanical force and the testis then digested with collagenase. Each pair of testes were incubated in a 50 ml pot (Falcon tissue culture flask, Becton Dickinson Labware, USA) containing an 8 ml solution of collagenase (Type I, Sigma Chemicals, UK) at a concentration of 0.25 mg/ml in Krebs-Ringer buffer containing bovine serum albumen (1% w/v) (BSA, fraction V, reagent grade Miles Laboratories, UK) and glucose (2%

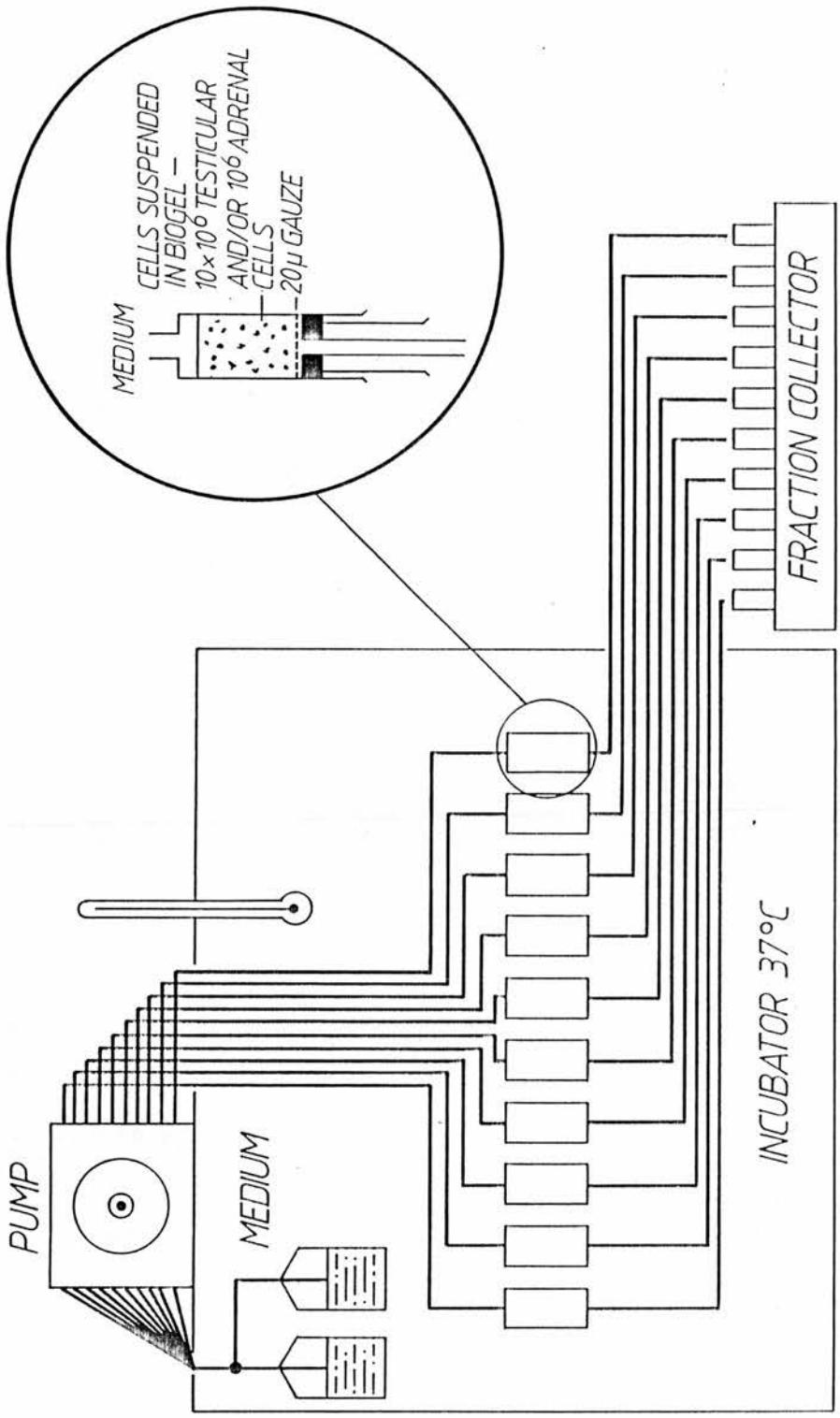


Figure 2.10: Diagram of multichannel isolated-cell superfusion system.

w/v) (AnalaR, British Drug Houses, UK) for 10 minutes in a shaking water bath at 37°C and 60 cycles per minute. The subsequent digest from the 10 testes was suspended in 100 ml Krebs-Ringer buffer in a 100 ml glass cylinder and allowed to sediment for 2 minutes by gravity. The supernatant containing isolated testicular cells was centrifuged (MSE Coolspin, Fisons UK) at 300 g for 15 minutes (Davies, 1979). The cell pellet was resuspended in buffer and 10 x 10^6 testicular cells packed into each superfusion chamber according to the experimental protocol. The preparation therefore represents a 'crude' preparation of cells that corresponds to approximately 10^6 Leydig cells as estimated by 3β-hydroxysteroid dehydrogenase activity (Browning, 1983; Sharpe, 1983b).

2.2.3: The superfusion system

The superfusion system employed a multichannel peristaltic pump (Desaga PLG, Uniscience, UK), an incubator (Gallenkamp, UK) and a fraction collector (2070 Ultrorac II, LKB, UK) (Figs 2.9 and 2.10). Each superfusion chamber contained 0.5 g of polyacrylamide gel (Bio-Gel, P-2, 200-400 mesh weight, Bio-Rad Laboratories, USA) based upon a 20 μ nylon mesh (Henry Simon Ltd, UK). Initial experiments used 2 ml disposable plastic syringes (Gillette Sabre, UK) as superfusion chambers. These syringes have the advantage of having a hollow plunger so that when a 21 FG needle (Microlance, Becton Dickinson, UK) is inserted into the bung and the 'female piece' of the needle is removed, a through route is created for superfusion. The syringes were assembled upside down and the 20 μ

nylon gauze is wrapped around the rubber bung to act as support for the Bio-Gel and cells (Fig 2.10). Bio-Gel was weighed dry and pre-wetted to form a slurry with 2.5 ml Krebs-Ringer buffer prior to loading into the syringe by suction and the gel allowed to settle while buffer was superfused through the chambers. Once the support of gel upon nylon gauze was established, isolated cells were loaded into the chamber by suction and the columns allowed to settle under superfusion for one hour prior to experimentation. Buffer was stored in the incubator during superfusion to maintain a constant temperature of 37°C and the medium constantly gassed with a mixture of 95% O_2 : 5% CO_2. However, later experiments used superfusion chambers manufactured from polytetrafluoroethylene (PTFE, Nylonic Engineering Co Ltd, Cumbernauld, Scotland) (McDougall, 1978) because non-polar steroids such as progesterone significantly adhere to the plastic syringes and silcone tubing.

Using ^{3}H-progesterone (Amersham plc, UK), only 5.3% of tracer was recovered from labelled Krebs-Ringer buffer that was superfused through a system of silicone tubing and Lowry columns, compared with 53% for silicone tubing and PFTE columns and over 80% (mean of 10 columns) for PFTE connecting tubing (silicone tubing is still retained for the pump) and PFTE columns. Using PFTE columns, PFTE connecting tubing and retaining silicone pump tubing, the mean $\pm$ SD (%) recovery of steroids (all at a concentration of 5 ng/ml) from the whole system was 86.3 $\pm$ 5.3 for progesterone, 89.1 $\pm$ 18.8 for 17α-hydroxyprogesterone, 78.3 $\pm$ 23.4 for androstenedione, 76.9 $\pm$ 13.9 for testosterone and 80.3 $\pm$ 22.9 for corticosterone (mean of

10 columns). Superfusion chambers were made from solid PFTE rods (0.5 and 1.0 inches in diameter) by Mr Louis Mackie, Chief Technician, Medical Physics Department, Radiotherapy Unit, Western General Hospital, Edinburgh. The pump tubing was made from silicone (Silicone C, internal diameter 1.0 mm and wall thickness 1.0 mm, Desaga PLG, Uniscience, UK) to deliver superfusion medium at a rate of 0.5 ml/min, with connecting tubing manufactured from PTFE (0.74 mm internal diameter, 0.254 mm wall and 0.051 mm tolerance, Jencons, UK) and stream dividers were used as necessary (Acculab, UK). Superfusion medium consisted of Krebs-Ringer buffer containing BSA (0.2% w/v) and glucose (0.2% w/v) at a temperature of 37°C maintained by the incubator and oxygenated with a mixture of oxygen 95% : 5% carbon dioxide (British Oxygen Company, UK) with or without stimuli. Isolated cells were stimulated by appropriate stimuli in Krebs-Ringer buffer for a period of 10 minutes followed by buffer alone for a period of 50 minutes (stimulus, control or buffer are introduced by means of a two-way tap) and superfusate samples collected every 5 minutes by the multichannel fraction collector for steroid hormone analysis. Samples were stored at -20°C prior to analysis by radioimmunoassay. Superfusion medium, Krebs-Ringer buffer containing glucose (0.2% w/v) and BSA (0.2% w/v), were made up on the morning of experimentation from the following stock solutions:

NaCl	326	ml 0.77 molar solution (45 g/l)
KCl	7	ml 0.77 molar solution (57.5 g/l)
$CaCl_2$	9.6	ml 0.55 molar solution (80.9 g $CaCl_2.2H_2O$/l)

KH_2PO_4 3.2 ml 0.77 molar solution (105.5 g/l)

$MgSO_4$ 3.2 ml 0.77 molar solution (189.8 g $MgSO_4.7H_2O$/l)

$NaHCO_3$ 336 ml 0.154 molar solution (12.9 g/l)

The volume was then made up to 2080 ml with distilled water, glucose and albumen added and the medium gassed with 95% O_2 : 5% CO_2 (BOC, UK) for 1 hour.

2.2.4: Stimuli for superfusion

i) ACTH: 1, 10, and 100 pg/ml of synthetic ACTH (Tetracosactrin, CIBA-Geigy Laboratories, Switzerland) in superfusion medium were prepared immediately prior to use and superfused over a 10 minute period according to the protocol.

ii) hCG: 50 ng/ml of human chorionic gonadotrophin (Pregnyl, Organon Laboratories Ltd, UK) in superfusion medium was also prepared immediately prior to use and superfused over a 10 minute period according to the protocol.

iii) Progesterone: 10 ng/ml of progesterone (Sigma Chemical Company, USA) in superfusion medium was also prepared immediately prior to use and superfused over a 10 minute period according to the protocol. Progesterone being a non-polar steroid was initially dissolved in absolute alcohol in the preparation of stock solutions and is subsequently serially diluted in alcohol until a concentration is achieved that is soluble in aqueous solution. The alcohol was then evaporated in a sample concentrator and the sample reconstituted in superfusion buffer and diluted to the concentrations employed in the experiments. At these

concentrations progesterone was readily maintained in aqueous solution. This procedure was necessary in order to prevent potential contamination of isolated cells with alcohol and subsequent cell damage.

2.2.5: Steroid hormone analysis for in vitro fluids

Steroid hormone analysis in buffer were performed by direct radioimmunoassay (either neat or in dilution) for progesterone (Ratcliffe, 1982), 17α-hydroxyprogesterone (Wallace, 1984), androstenedione, testosterone (Corker, 1978) and corticosterone (Al-Dujaili, 1981b), all employing ^{125}I-radioligands. All the assays wre adapted from existing plasma assays to be performed in superfusion buffer.

2.2.5.1: In vitro progesterone assay

For direct assay, 200 µl of sample (either neat or in dilution 1:5 in superfusion medium) or standard (over the range 0.156 to 40 ng/ml in superfusion medium) was added to 100 µl of antibody (R31/7 as for the in vivo progesterone assay, initial dilution 1:6000; final dilution 1:30,000) and 200 µl of tracer (^{125}I-progesterone as for the in vivo progesterone assay, 10,000 counts per minute), all in EDTA-phosphate assay-buffer (pH 7.4). Samples were incubated in borosilicate tubes 10 x 75 mm (Corning, USA) at room temperature for 4 hours prior to addition of second antibody. Precipitation of tracer bound to antibody was achieved with second antibody, consisting of 50 µl donkey anti-rabbit antiserum (initial dilution

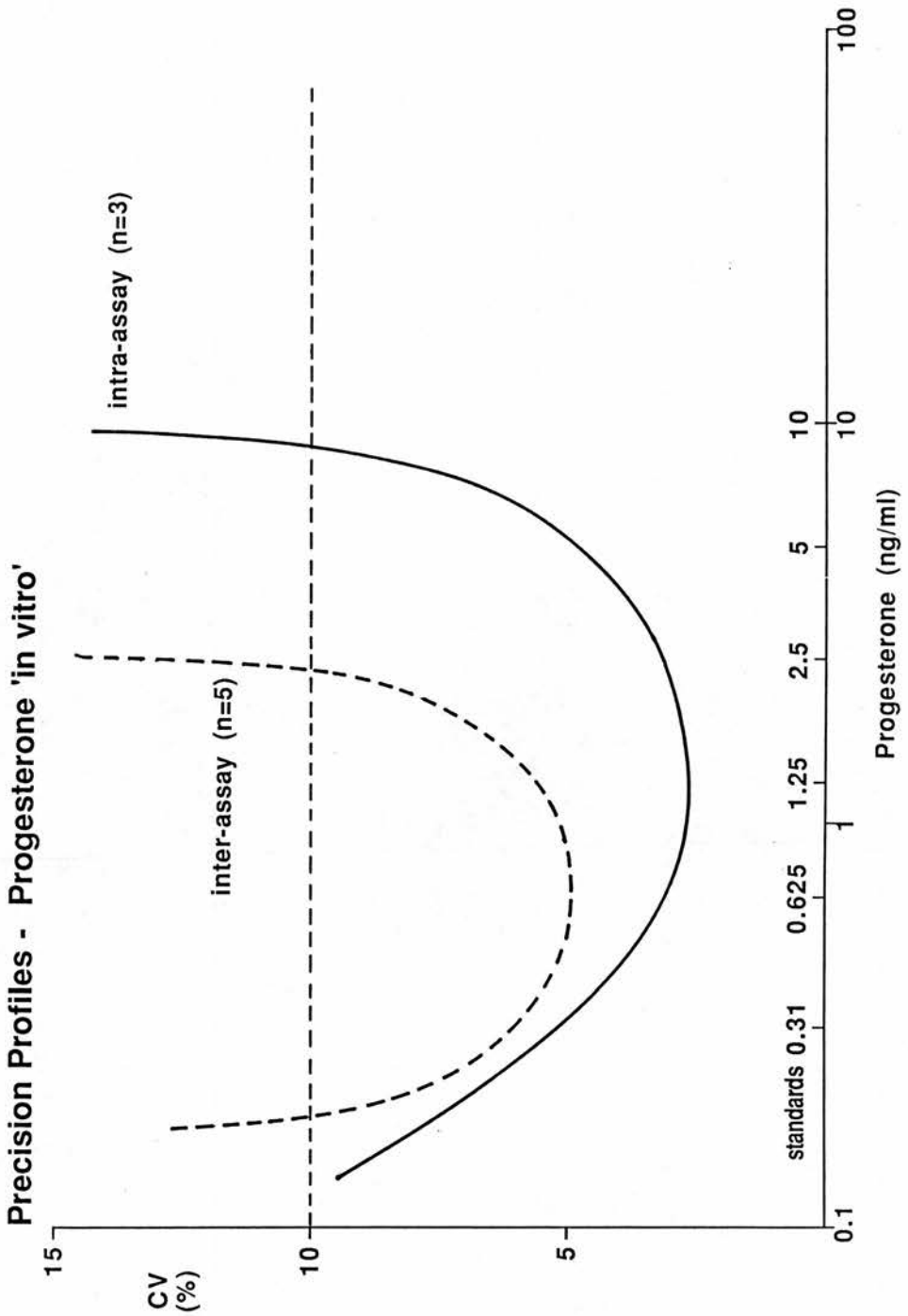
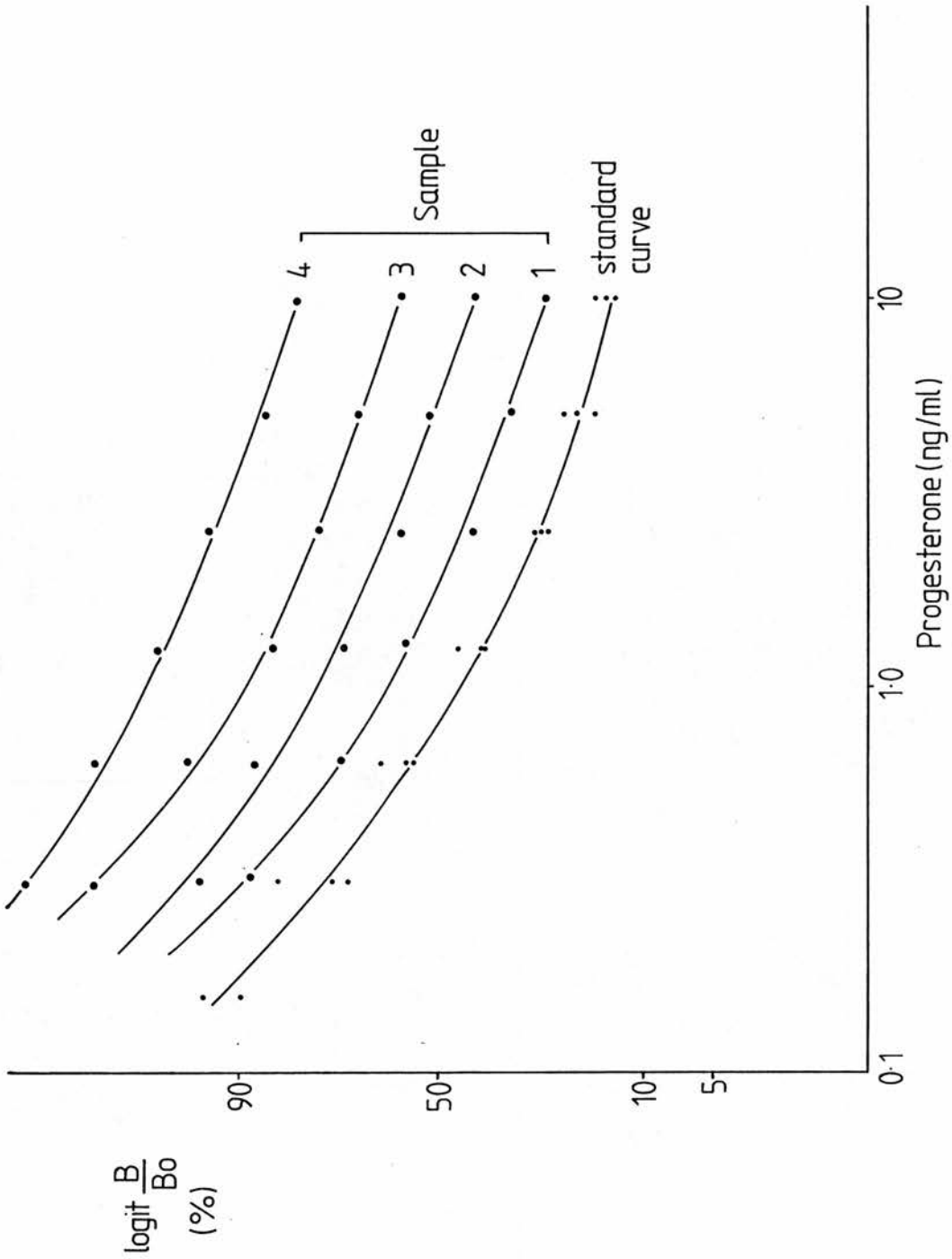


Figure 2.11: Precision profiles for <u>in vitro</u> progesterone assay.



Note: dilution of plasma samples is shown transposed from the standard curve to illustrate parallelism.

Figure 2.12: A typical standard curve for <u>in vitro</u> progesterone samples with dilutional studies.

Figure 2.13: Cross-reactivities of progesterone antibody to possible interfering steroids.

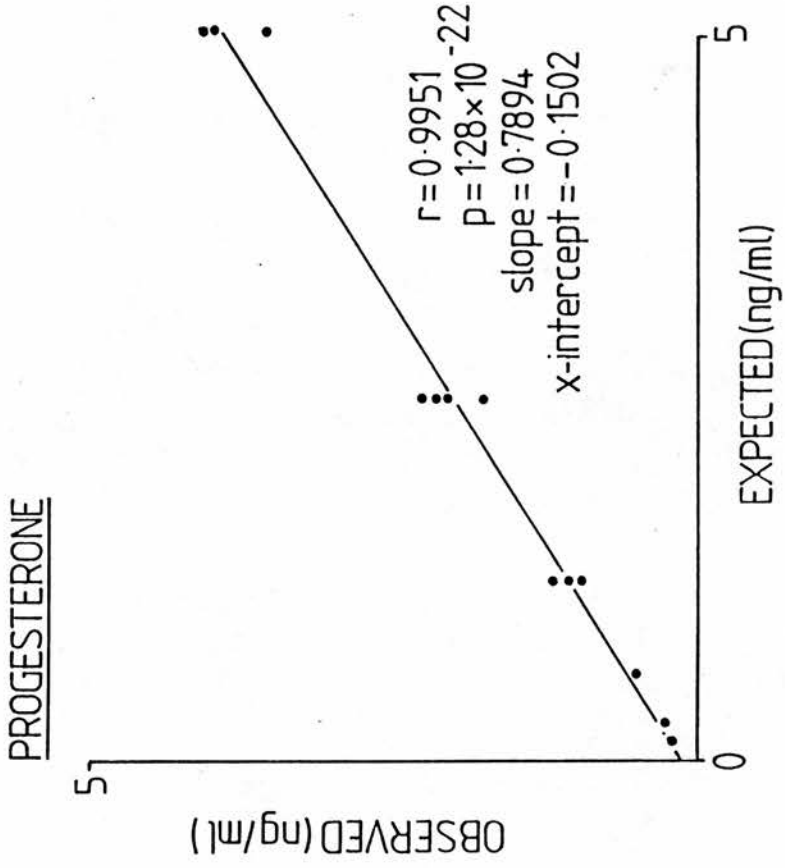


Figure 2.14: Recovery studies for progesterone from in vitro samples.

1:10; final dilution 1:120) and 50 µl normal rabbit serum (initial dilution 1:100; final dilution 1:1200), provided by the Scottish Antibody Production Unit (Carluke, Scotland). Following addition of second antibody, samples were incubated overnight at 4°C and then centrifuged (MSE, Coolspin, Fisons, UK) at 2000 g for 30 minutes at 4°C, the supernatant decanted and the precipitate counted in a gamma counter (LKB-Wallac, UK).

Profiles of intra- and inter-assay precision are illustrated in figure 2.11. Over the working range 0.156 to 5 ng/ml intra- and inter-assay precisions are respectively 8.6% and 12.5% at 0.156 ng/ml and 4.5% and 27% at 5 ng/ml. The working range was usually determined by the limits 90-10% B/B_0 (see figure 2.12 for a typical standard curve) as well as the precision profile.

Double dilution of samples in superfusion buffer produced dilutional curves parallel to the standard curve (Fig 2.12) and cross-reactivities (Fig 2.13) to possible interfering steroids are in close agreement with those previously published with significant cross-reactivity to 17α-hydroxyprogesterone (15%) and 11-deoxycortisol (3.0%).

Recovery of progesterone (observed) from superfusion medium is in close agreement with that expected (Fig 2.14).

2.2.5.2: In vitro 17α-hydroxyprogesterone assay

For assay, 100 µl of sample (either neat or diluted 1:5 in superfusion medium) or standard (over the range 0.05 to 3.0 ng/ml in superfusion medium) was added to 200 µl of antibody (raised in

rabbits against 17α-hydroxyprogesterone-3-(-O-carboxymethyl)-oxime-BSA and microencapsulated, initial dilution 1:40; final dilution 1:200) and 200 µl of tracer (17α-hydroxyprogesterone-3-(-O-carboxymethyl)-oxime-^{125}I-histamine) both in 0.5 molar phosphate assay-buffer (pH 7.4) and incubated in plastic LP4 tubes (Luckham Ltd, UK) at room temperature for 45 minutes. Separation of tracer bound to antibody was achieved by washing twice with 2 ml saline (0.9% w/v) containing polyoxyethylene(20)sorbitan monolaurate (Sigma Chemicals, USA) (0.5% v/v) as a detergent. After each wash, tubes were centrifuged at 1500 g for 15 minutes and the supernatant discarded. The remaining pellet, bound tracer, was counted in a gamma counter (LKB-Wallac, UK). The microencapasulated 17-hydroxyprogesterone antibody was a gift from Dr A M Wallace, Department of Clinical Chemistry, Royal Infirmary, Glasgow and tracer was prepared by coupling 17α-hydroxyprogesterone-3-(-O-carboxymethyl)oxime to ^{125}I-histamine (see section 2.2.5.6). 0.5 molar phosphate assay-buffer (pH 7.4) consisted of:

Na_2HPO_4.anhydrous (7.1 g/l)

KH_2PO_4 (6.9 g/l)

Both reagents were available from AnalaR, BDH, UK and were dissolved separately, each in 1 litre distilled water. 100 to 200 ml of KH_2PO_4 was then added to the solution of Na_2HPO_4 until the pH adjusted to 7.4. Following this, BSA (0.1% w/v) (fraction V, Miles Reagents, UK) was dissolved into solution and stored for use at 4°C.

Profiles of intra- and inter-assay precision are illustrated

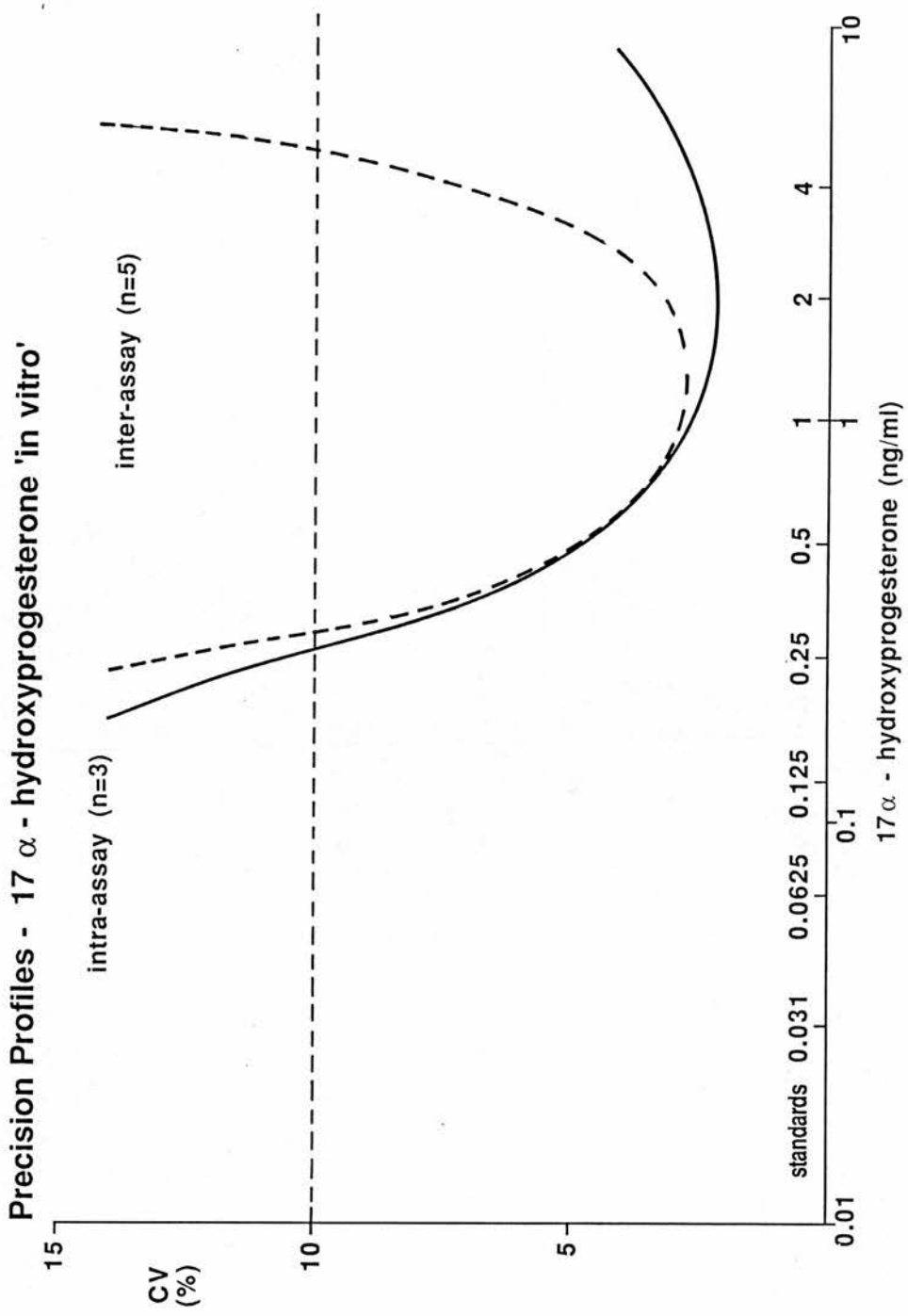


Figure 2.15: Precision profiles for <u>in vitro</u> 17α-hydroxy-progesterone assay.

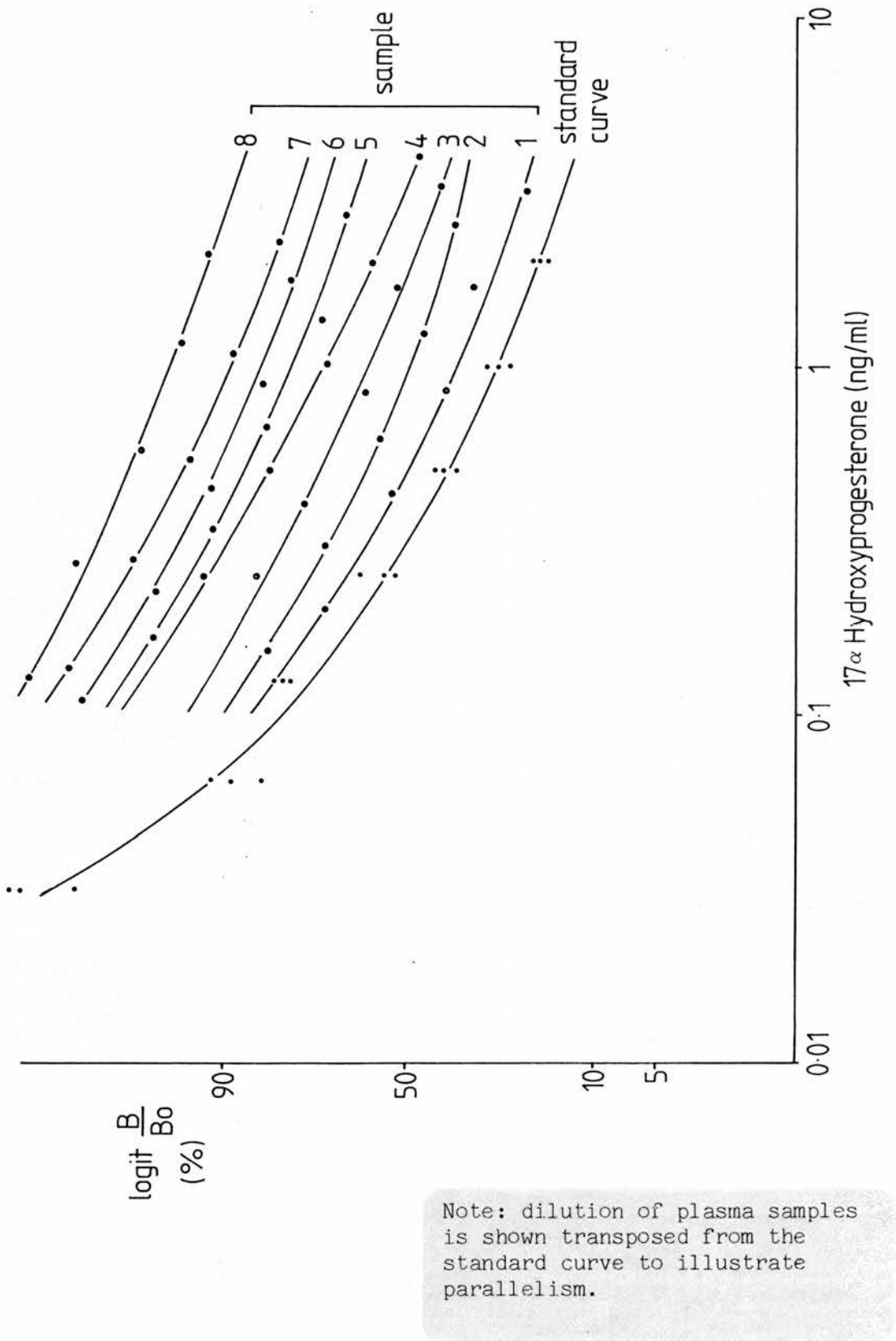


Figure 2.16: A typical standard curve for <u>in vitro</u> 17α-hydroxyprogesterone samples with dilutional studies.

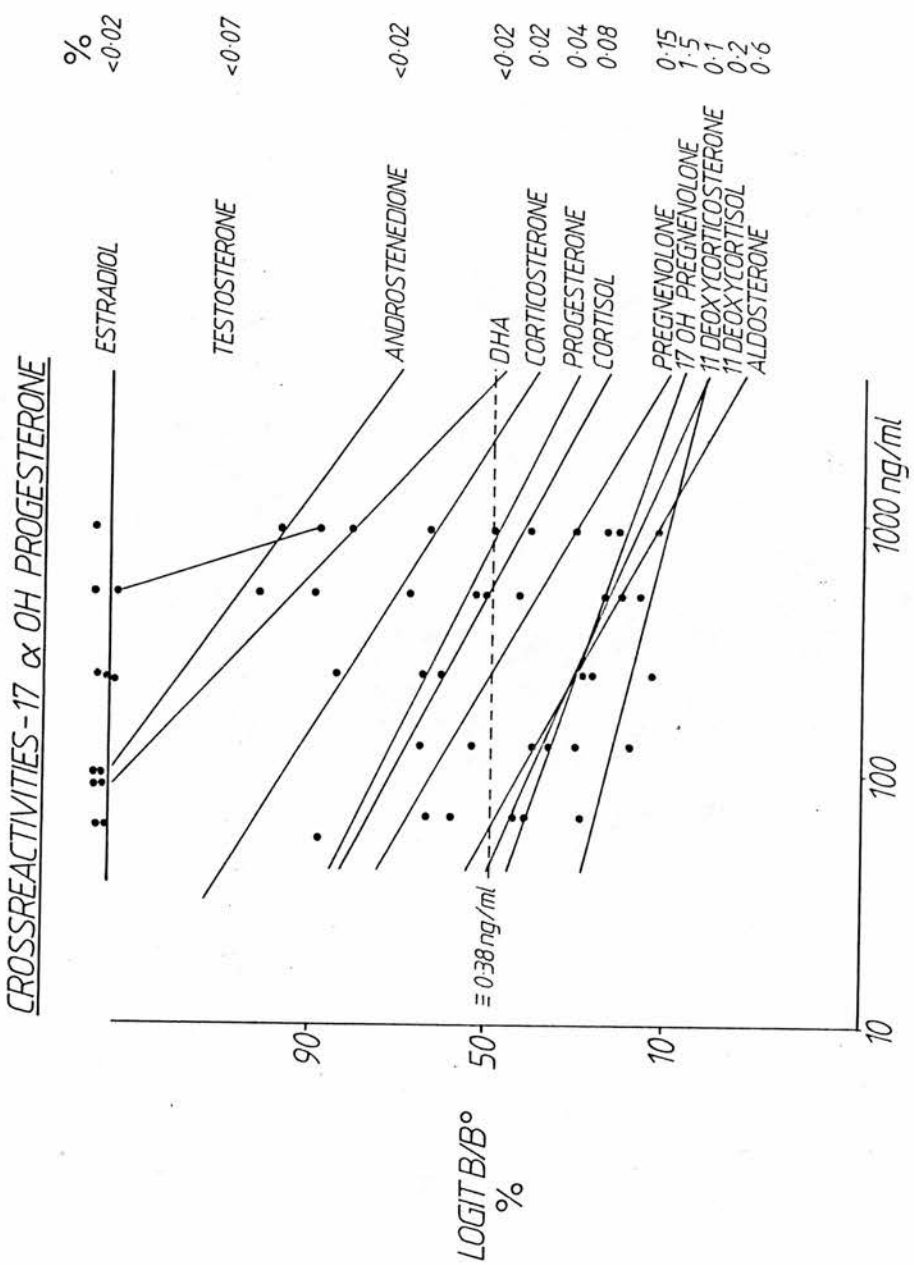


Figure 2.17: Cross-reactivities of 17α-hydroxyprogesterone antibody to possible interfering steroids.

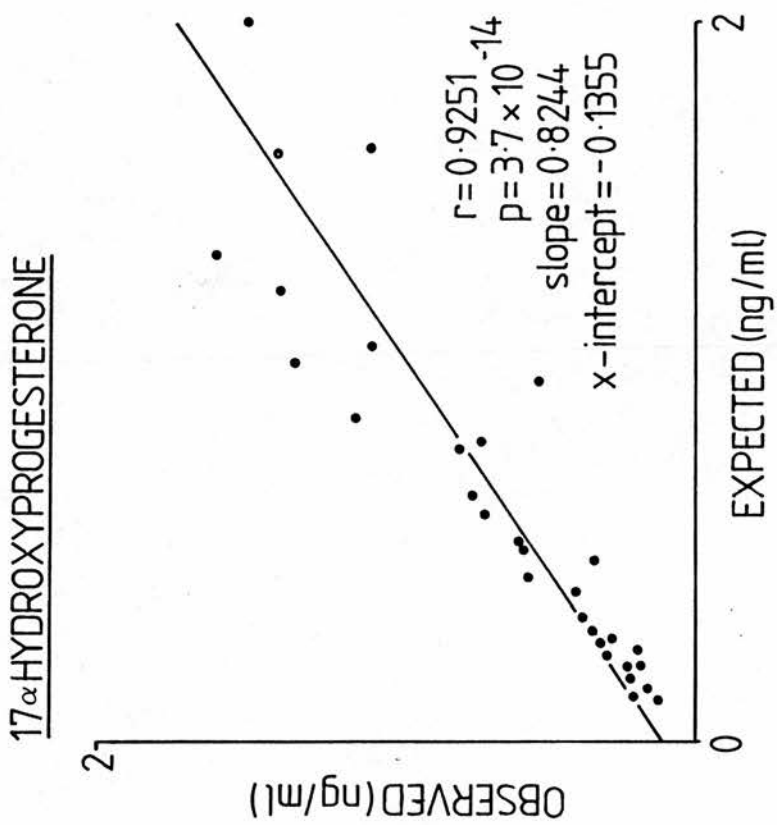


Figure 2.18: Recovery studies for 17α-hydroxyprogesterone from in vitro samples.

in figure 2.15. Over the working range 0.25 to 4 ng/ml the intra- and inter-assay precisions are respectively 10% and 13% at 0.25 ng/ml and 2.5% and 10% at 4 ng/ml. The working range was usually determined by the limits 90-10% B/B_0 (see figure 2.16 for a typical standard curve) as well as the precision profile.

Double dilution of samples in superfusion buffer produced dilution curves parallel to the standard curve (Fig 2.16) and cross-reactivities to possible interfering steroids (Fig 2.17) are in close agreement with those previously published with significant cross-reactivity to 17-hydroxypregnenelone (1.5%).

Recovery of 17α-hydroxyprogesterone (observed) from superfusion medium is close to that expected (Fig 2.18).

2.2.5.3: In vitro androstenedione assay

For assay, 100 µl of sample (either neat or diluted 1:5 in superfusion medium) or standard (over the range 0.031 to 2 ng/ml in superfusion medium) was added to 100 µl of antibody (Guildhay, HP/S/ 673-IA, raised in sheep against androstenedione-7 - carboethylthioether-ovalbumen, initial dilution 1:90,000; final dilution 1:270,000) and 100 µl of tracer (androstenedione-3-(-O-carboxymethyl)-oxime-(^{125}I)-iodohistamine, 10,000 counts per minute), all in 0.5 molar phosphate assay-buffer (pH 7.4). Samples were incubated in plastic LP3 tubes (Luckhams Ltd, UK) at 4°C overnight prior to separation with the addition of 1 ml dextran-coated charcoal. Samples were immediately centrifuged (MSE Coolspin, Fisons, UK) at 2000 g at 4°C for 15 minutes. The

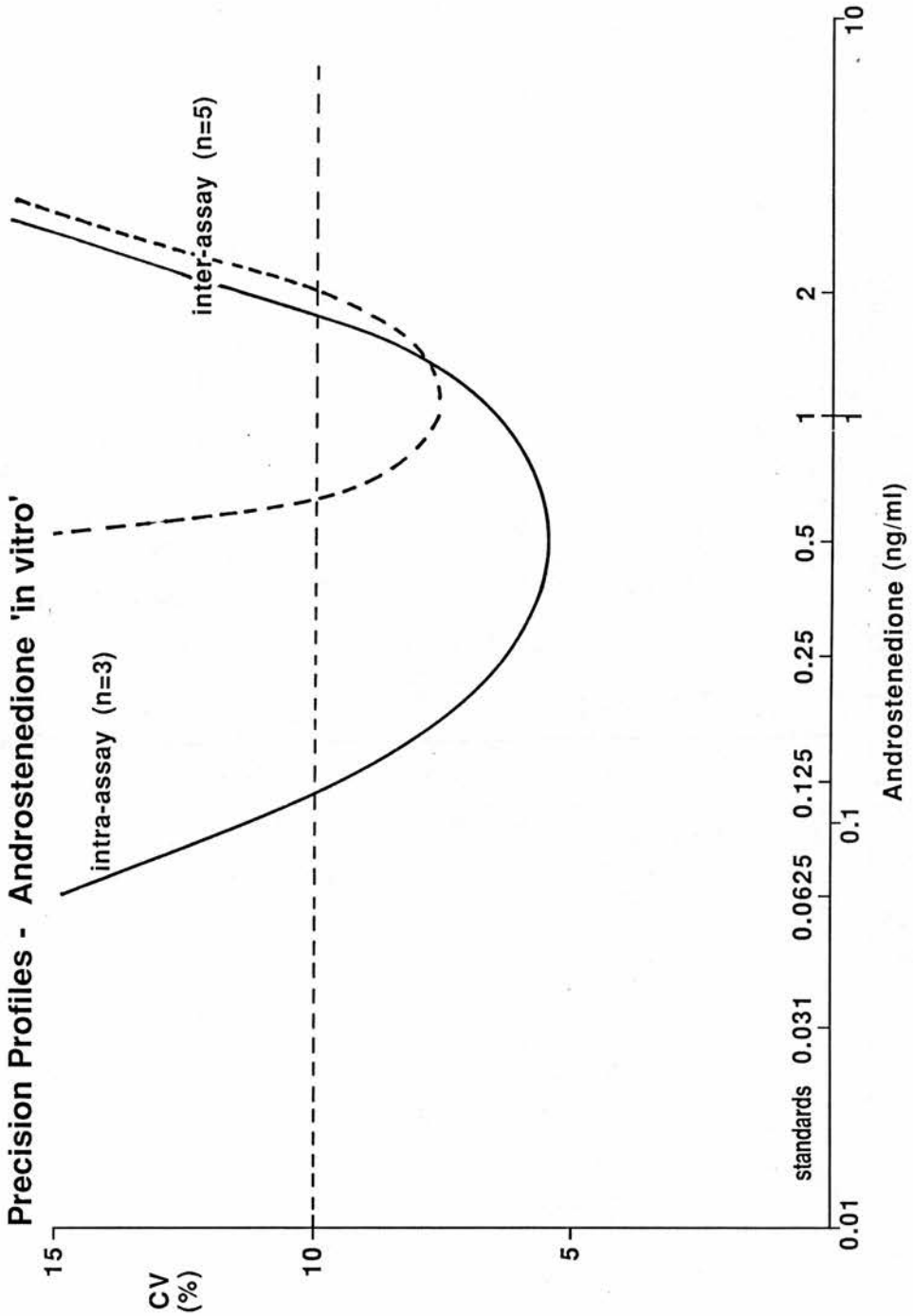


Figure 2.19: Precision profiles for <u>in vitro</u> androstenedione assay.

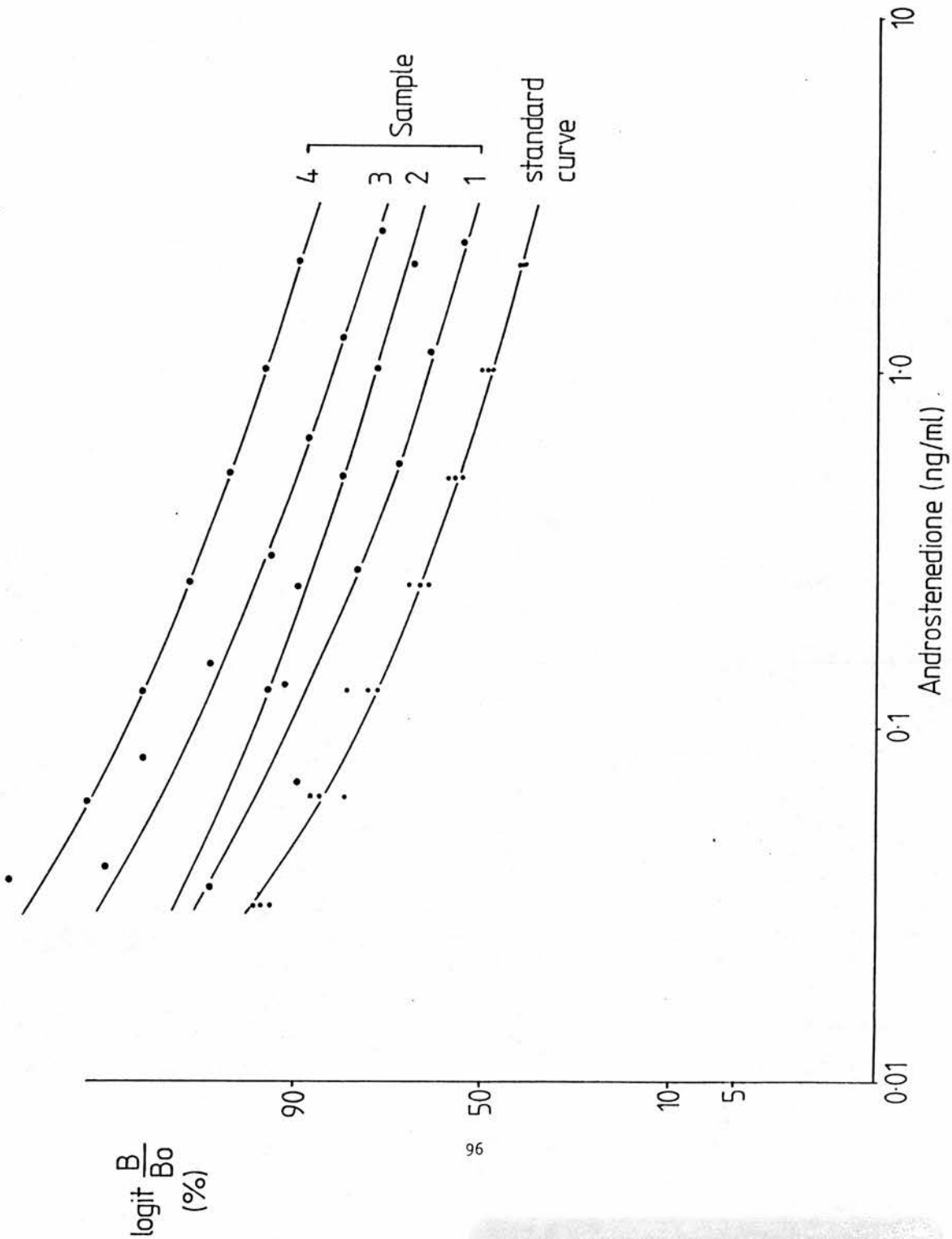


Figure 2.20: A typical standard curve for <u>in vitro</u> androstenedione samples with dilutional studies.

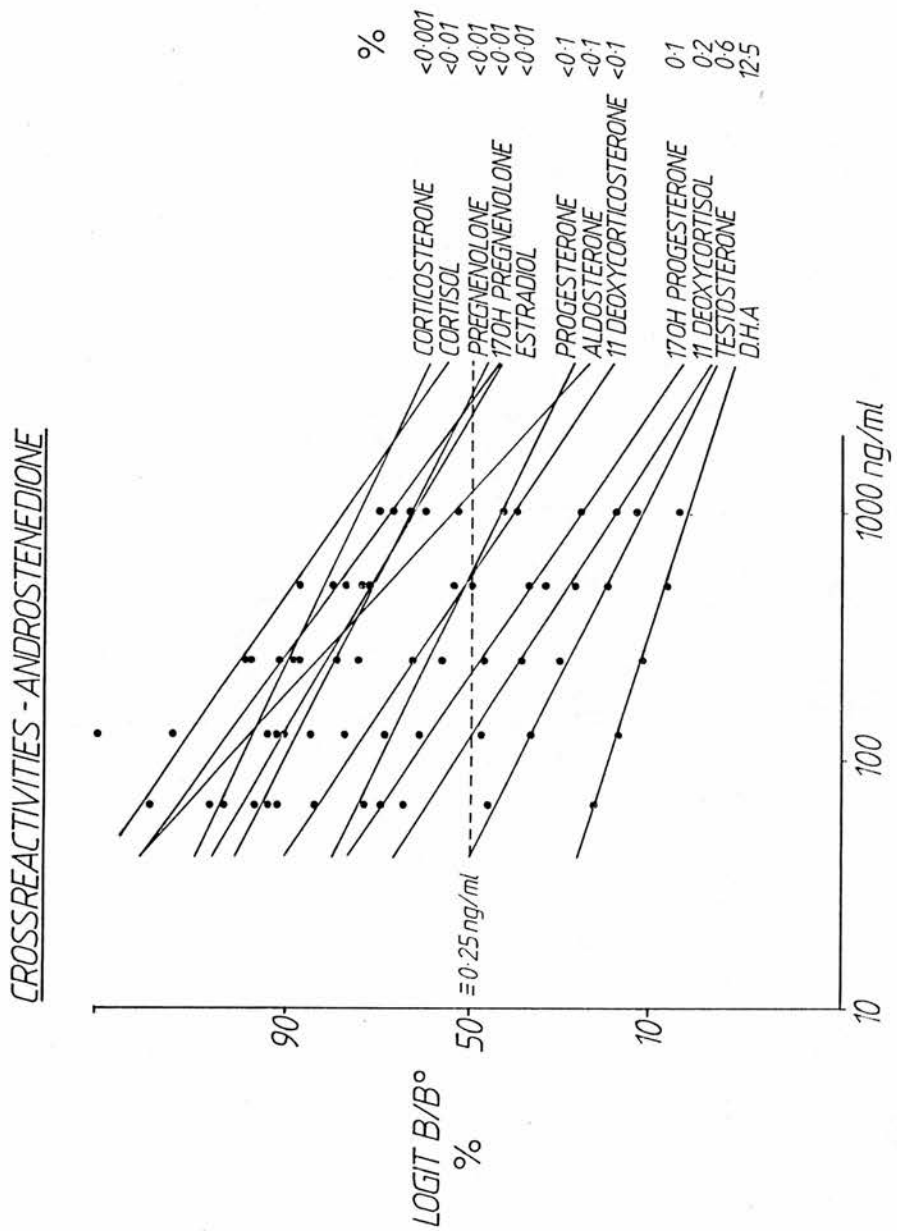


Figure 2.21: Cross-reactivities of androstenedione antibody to possible interfering steroids.

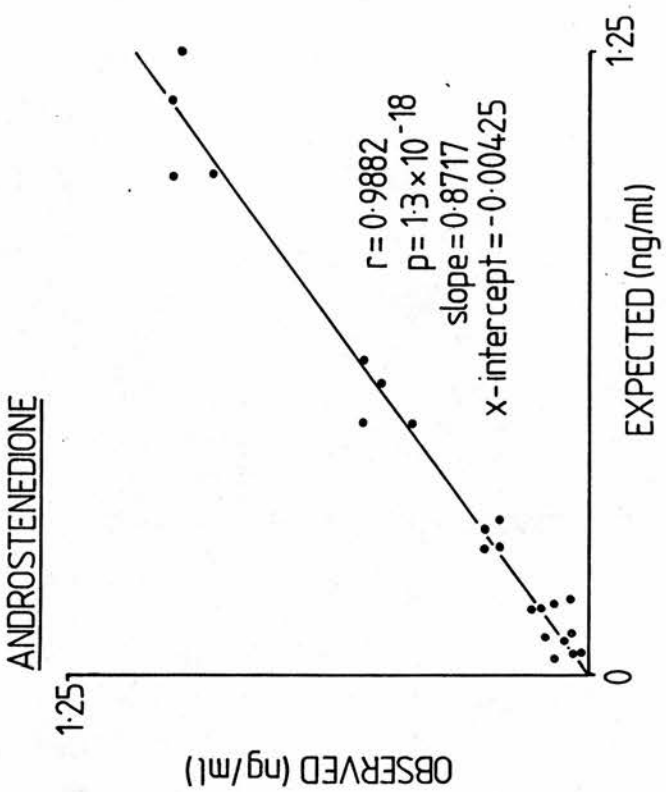


Figure 2.22: Recovery studies for androstenedione in <u>in vitro</u> samples.

supernatant was aspirated, discarded and the remaining pellet, unbound (free) tracer, counted in a gamma counter (LKB-Wallac, UK). The antibody was supplied by Guildhay Antisera, University of Surrey and the tracer was prepared by coupling androstenedione-3-(-O-carboxymethyl)-oxime with ^{125}I-histamine (see section 2.2.5.6).

Profiles for intra- and inter-assay precision are illustrated in figure 2.19. Over the working range of 0.25 to 2 ng/ml the intra- and inter-assay precisions are respectively 6.4% and 17% at 0.25 ng/ml and 10% and 11.5% at 2 ng/ml. The working range was usually determined by the limits 90-10% B/B_0 (see figure 2.20 for a typical standard curve) as well as the precision profile.

Double dilution of samples in superfusion buffer produced dilution curves parallel to the standard curve (Fig 2.20) and cross-reactivities to possible interfering steroids (Fig 2.21) are in close agreement with those previously published with significant cross-reactivity to dehydroepiandrosterone (12.5 %), testosterone (0.6%) and 11-deoxycortisol (0.2%).

Recovery of androstenedione (observed) from superfusion medium is close to that expected (Fig 2.22).

2.2.5.4: In vitro testosterone assay

For assay, 100 µl sample (either neat or diluted 1:5 in superfusion medium) or standard (over the range 0.025 to 3.2 ng/ml in superfusion medium) was added to 100 µl antibody (E01, as for *in vitro* assay, initial dilution 1:10,000; final dilution 1:30,000) and 100 µl of tracer (testosterone-3-(-O-carboxymethyl)-oxime-^{125}I-

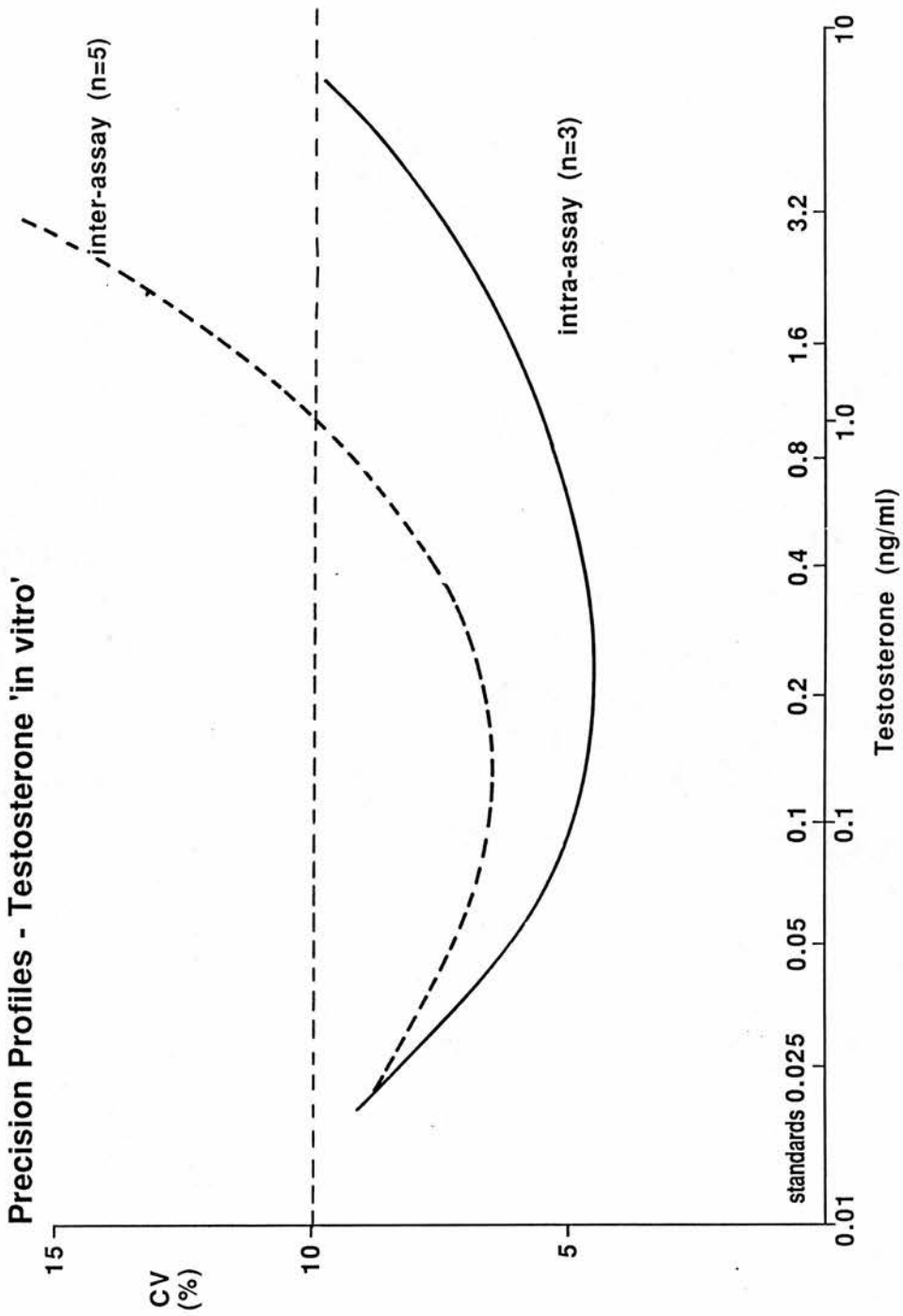


Figure 2.23: Precision profiles for <u>in vitro</u> testosterone assay.

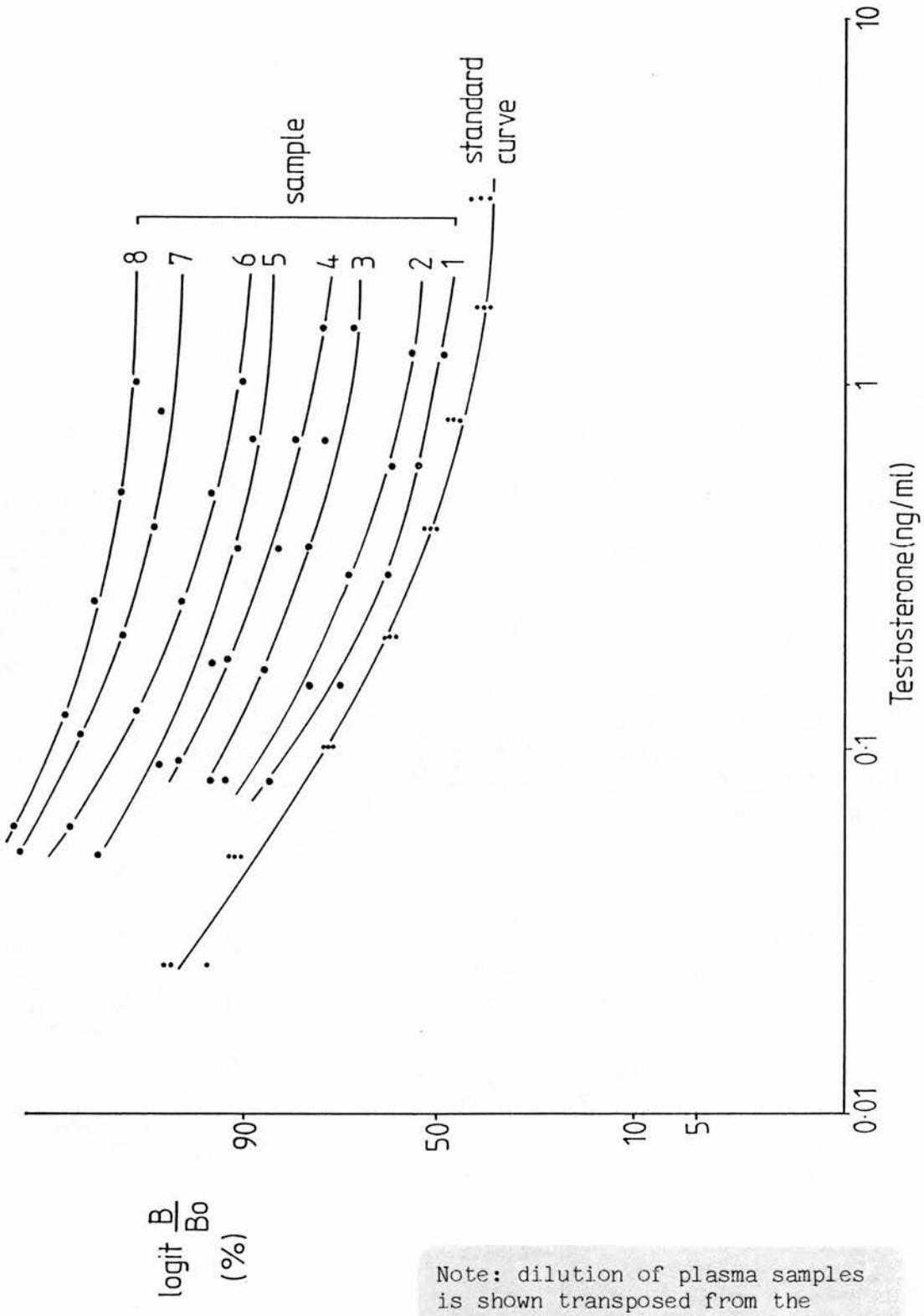


Figure 2.24: A typical standard curve for testosterone samples in vitro with dilutional studies.

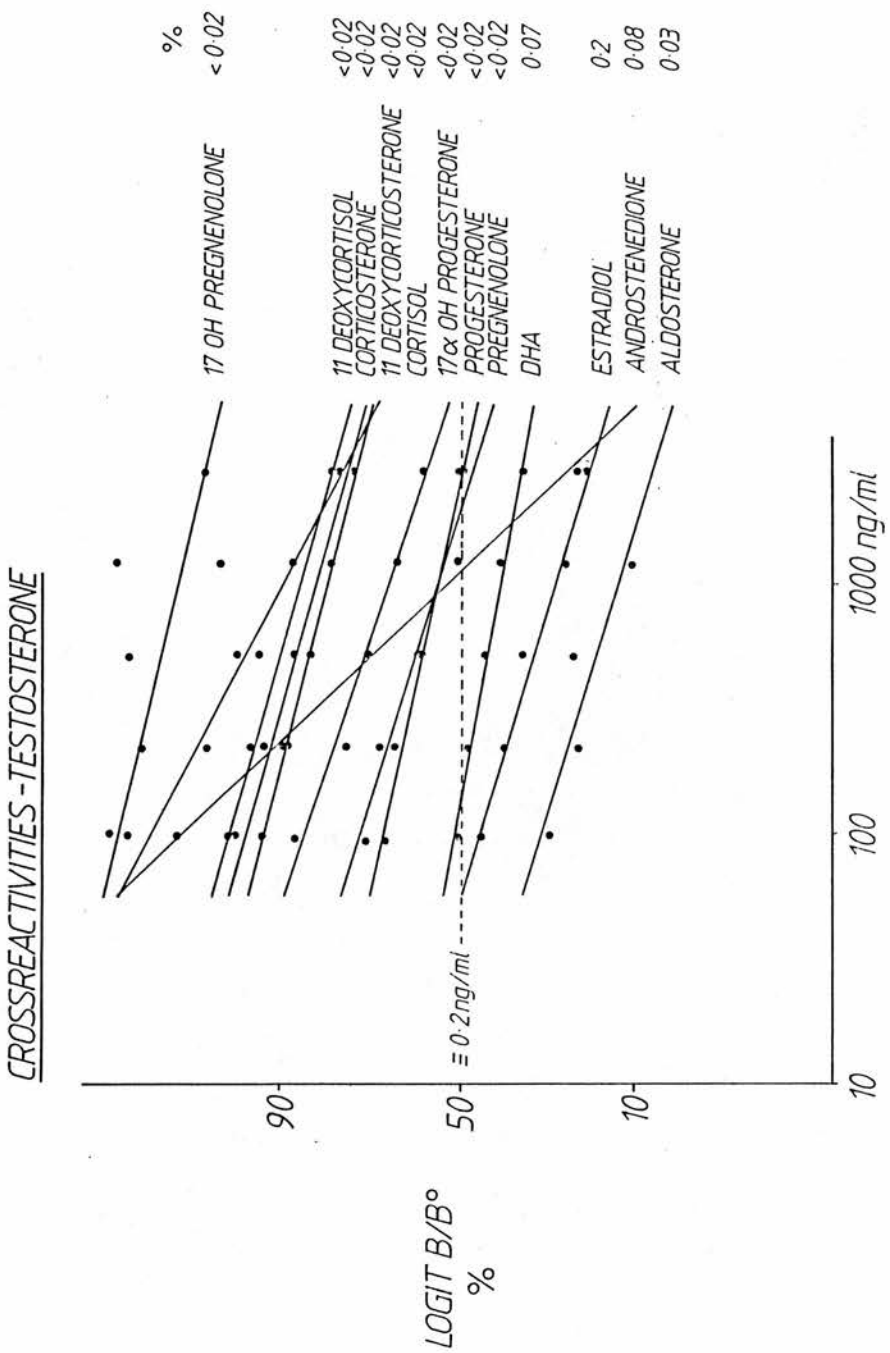


Figure 2.25: Cross-reactivities of testosterone antibody to possible interfering steroids.

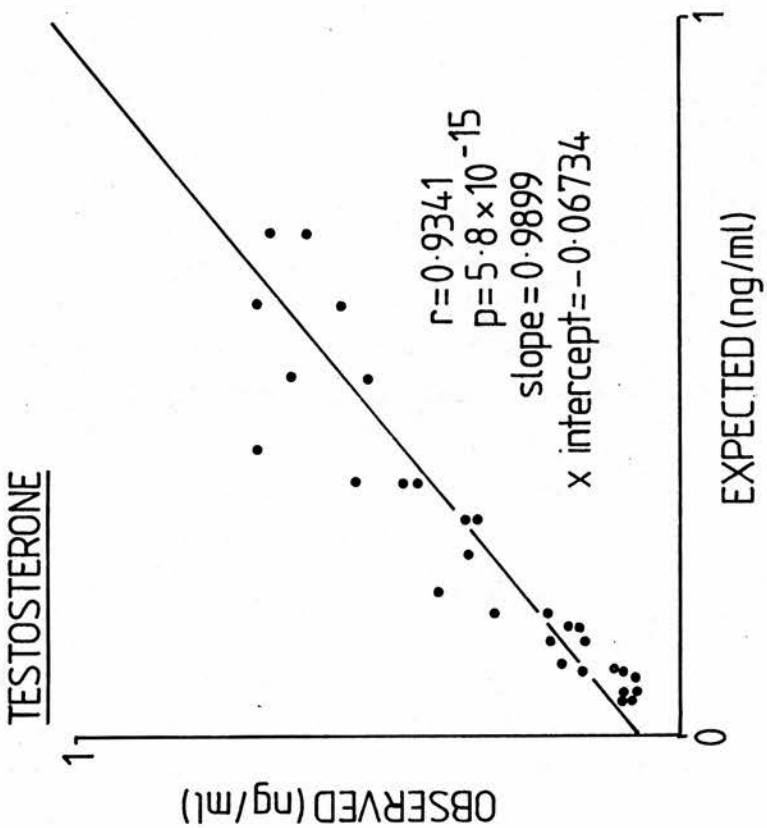


Figure 2.26: Recovery studies for testosterone in <u>in vitro</u> samples.

iodohistamine, 3000 counts per minute), both in 0.5 molar phosphate assay-buffer. Assays were incubated in plastic LP3 tubes (Luckhams Ltd, UK) overnight at 4°C prior to separation with 1 ml of dextran-coated charcoal and centrifuged at 2000 g (MSE Coolspin, Fisons, UK) at 4°C. The supernatant was aspirated and the remaining pellet, unbound tracer (free), counted in a gamma counter (LKB-Wallac, UK).

Profiles of intra- and inter-assay precision are illustrated in figure 2.23. Over the working range 0.025 to 3.2 ng/ml intra- and inter-assay precisions are respectively 8% and 8.5% at 0.025 ng/ml and 7.5% and 15.5% at 3.2 ng/ml. The working range was usually determined by the limits 90-10% B/B_0 (see figure 2.24 for a typical standard curve) as well as the precision profile.

Double dilution of samples in superfusion buffer produced dilutional curves parallel to the standard curve (Fig 2.24). Cross-reactivities (Fig 2.25) with possible interfering steroids are in close agreement with those previously published with significant cross-reactivities to dihydrotestosterone (10%) and androstenedione (0.08%).

Recovery of testosterone (observed) from superfusion medium is close to that expected (Fig 2.26).

2.2.5.5: In vitro corticosterone assay

For assay, 100 µl sample (either neat or in dilution at 1:10 or 1:100 in superfusion medium) or standard (over the range 0.5 to 64 ng/ml in superfusion medium) was added to 100 µl of antibody

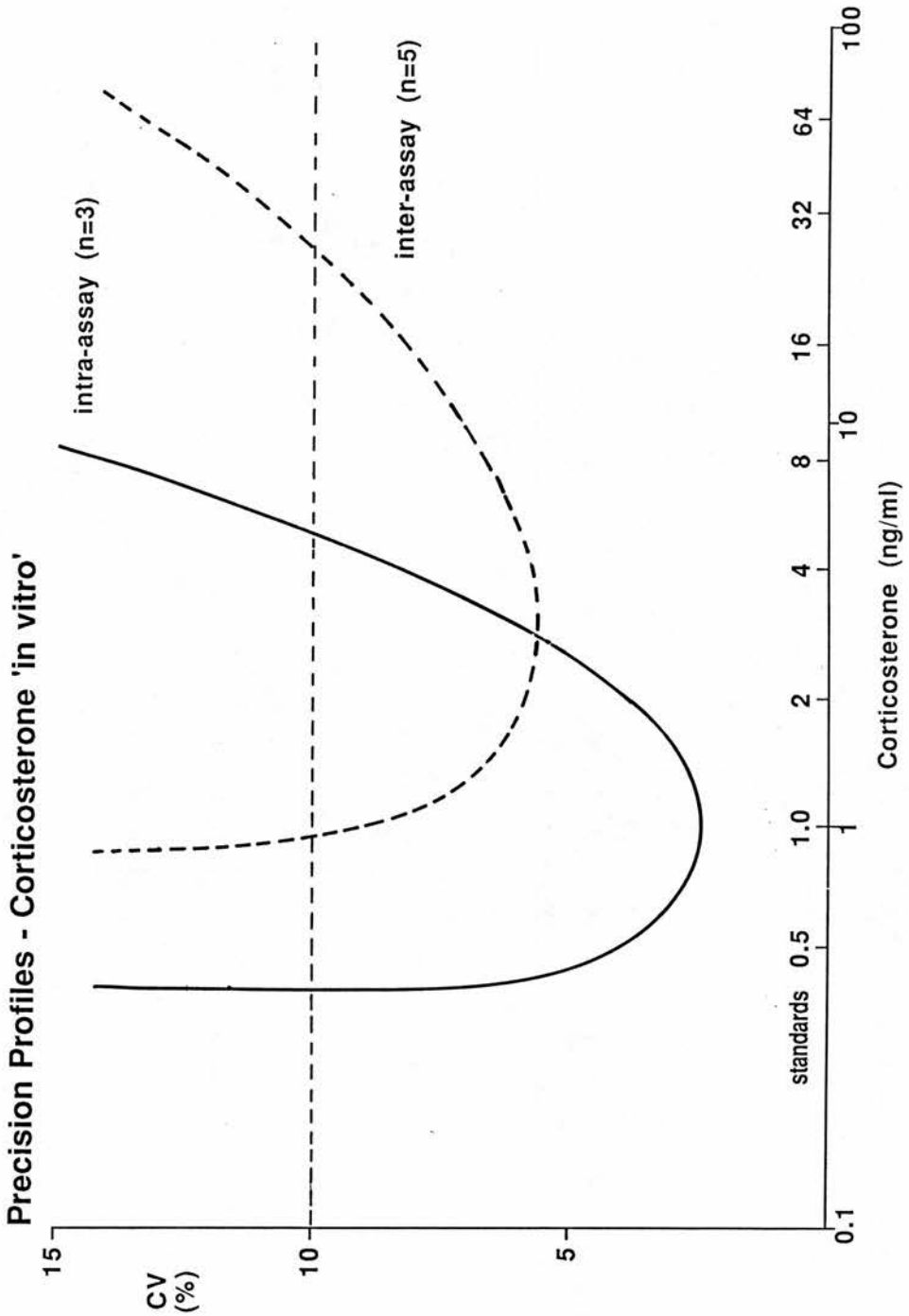
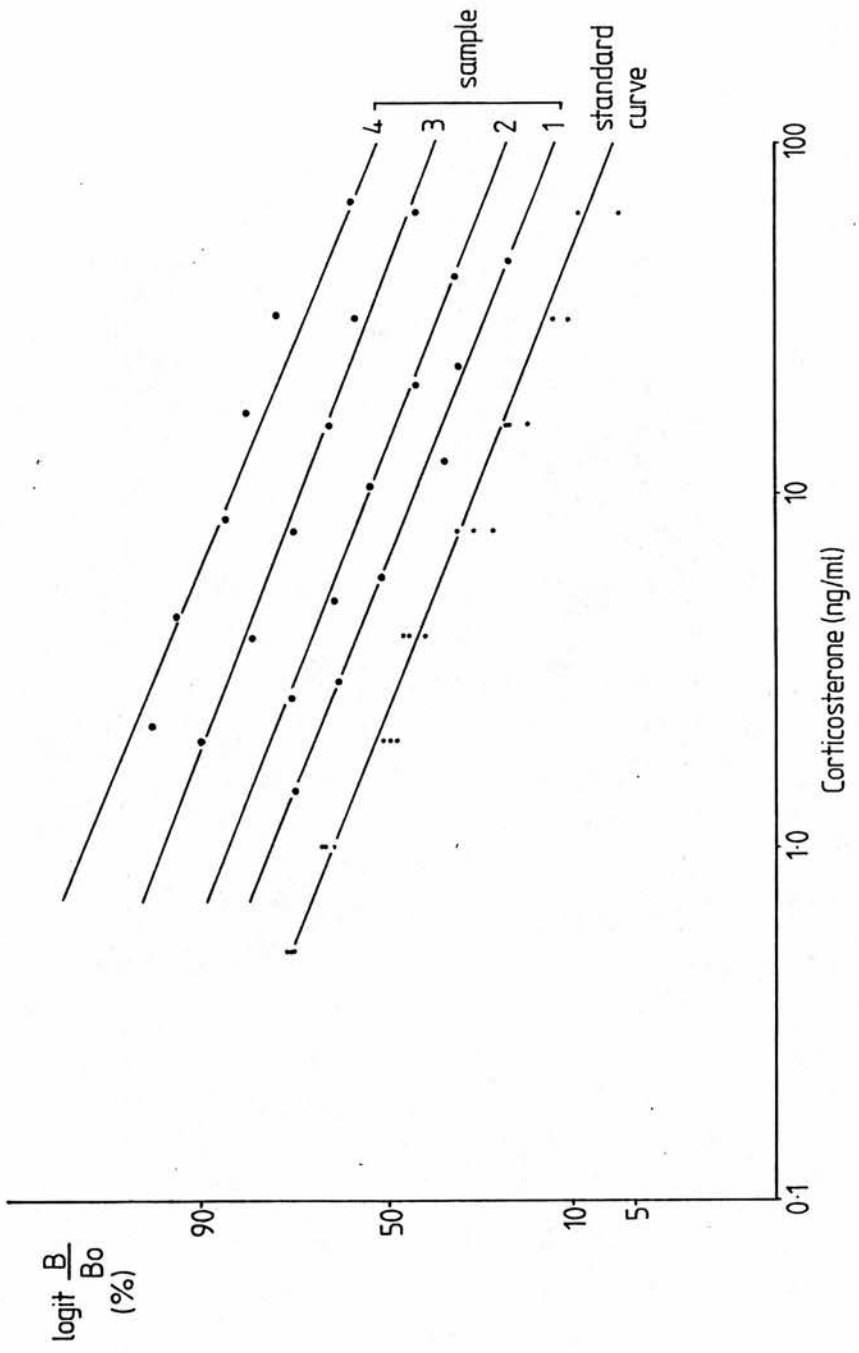


Figure 2.27: Precision profiles for <u>in vitro</u> corticosterone assay.



Note: dilution of plasma samples is shown transposed from the standard curve to illustrate parallelism.

Figure 2.28: A typical standard curve for <u>in vitro</u> corticosterone samples with dilutional studies.

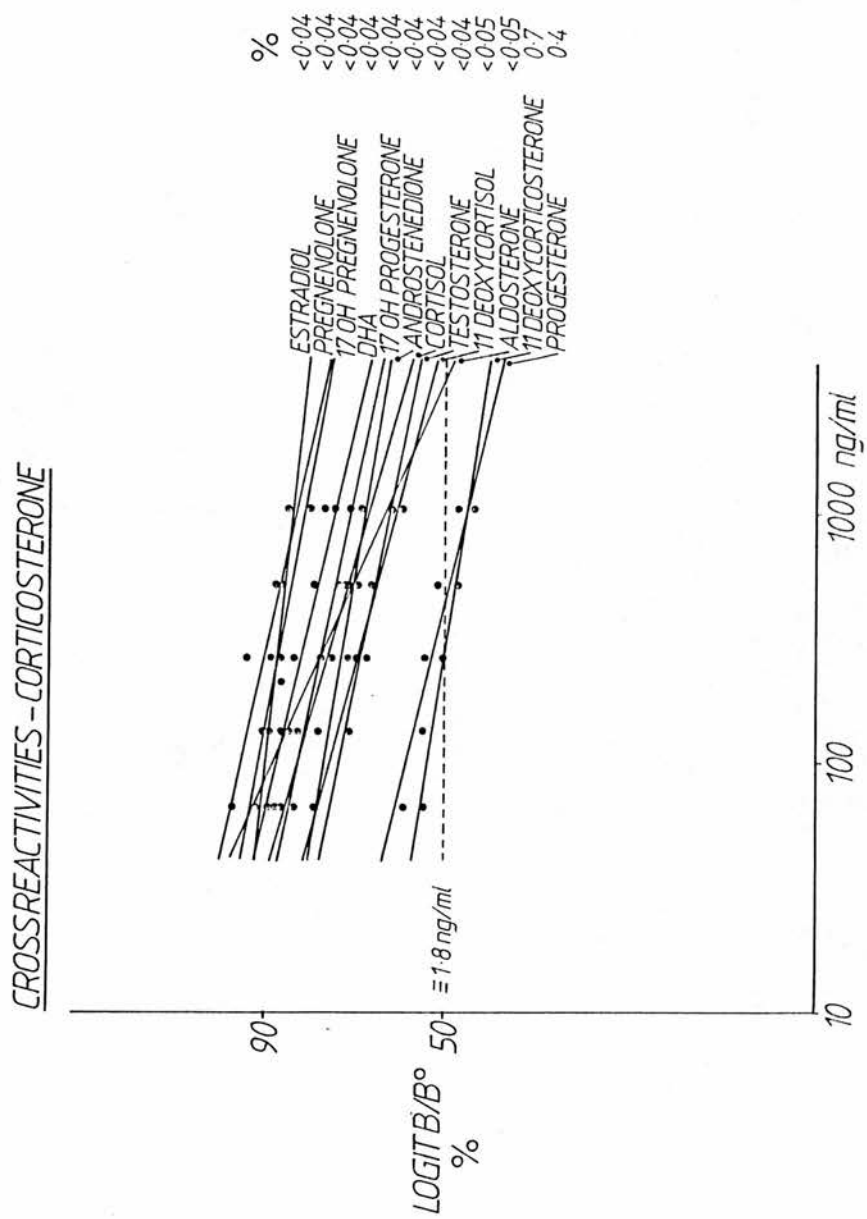


Figure 2.29: Cross-reactivities of corticosterone antibody to possible interfering steroids.

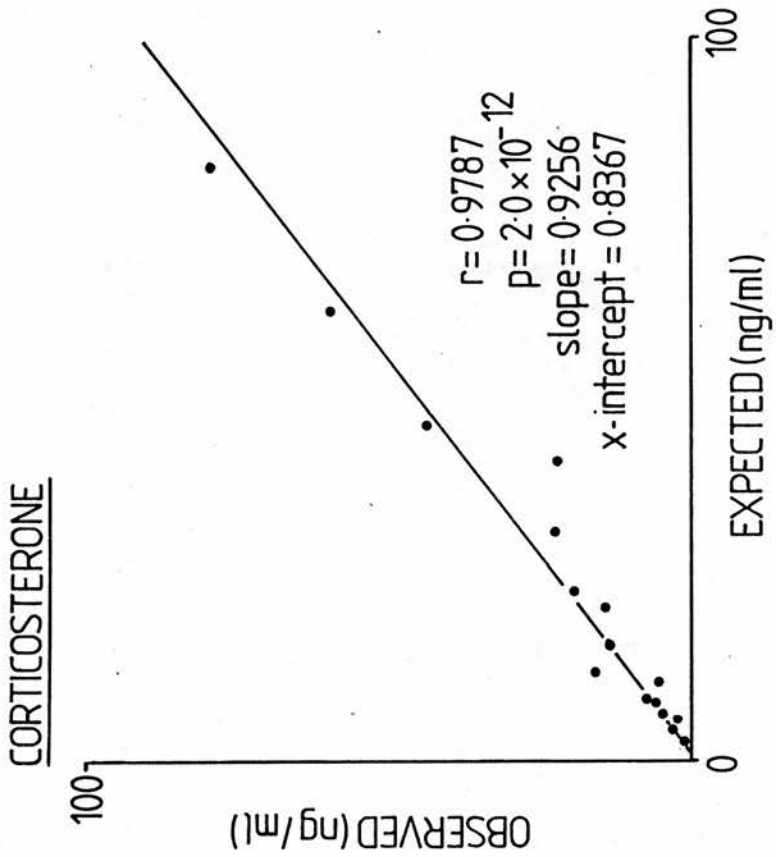


Figure 2.30: Recovery studies for corticosterone from in vitro samples.

(R1/B3 as for in vivo assay, initial dilution 1:15,000; final dilution 1:45,000) and 100 µl of tracer (corticosterone-3-(O-carboxymethyl)-oxime-^{125} I-histamine 3000 counts per minute), both in 0.5 molar phosphate assay-buffer. Assays were incubated in plastic LP3 tubes (Luckhams Ltd, UK) overnight at 4°C prior to separation with 1 ml dextran-coated charcoal and centrifuged (MSE, Coolspin, Fisons, UK) at 2000 g at 4°C for 15 minutes. The supernatant was aspirated, discarded and the remaining pellet, unbound (free) tracer, was counted in a gamma counter (LKB-Wallac, UK).

Profiles of intra- and inter-assay precision are illustrated in figure 2.27. Over the working range 0.5 to 16 mg/ml the intra- and inter-assay precisions are are respectively 4.5% and 17.2% at 0.5 ng/ml and 8.5% and 20% at 16 ng/ml. The working range was usually determined by the limits 90-10% B/B_0 (see figure 2.28 for a typical standard curve) as well as the precision profile.

Double dilution of samples in superfusion medium produced dilutional curves parallel to the standard curve (Fig 2.28) and cross-reactivities (Fig 2.29) with possible interfering steroids are in close agreement with those previously published with significant cross-reactivity to 11-deoxycorticosterone (0.7%).

Recovery of corticosterone (observed) from superfusion medium is close to that expected (Fig 2.30).

2.2.5.6: Preparation of tracers

Tracers for 17α-hydroxyprogesterone, androstenedione, testosterone and corticosterone assays were prepared by coupling the

steroid-3-(-O-carboxymethyl)-oxime with ^{125}I-histamine by the chloramine-T technique (Greenwood, 1963). Iodination of the steroid took place in three stages in a fume cupboard:

i) **Steroid activation**

200 µg of steroid-3-(-O-carboxymethyl)-oxime (Steraloids, UK) was dissolved in 100 µl of dioxan in a glass tube prior to the addition of 10 µl tri-n-butylamine (20% in dioxan v/v) and 10 µl isobutylchlorformate (10% in dioxan v/v) (reagents BDH, UK). The mixture was vortexed and incubated for 30 minutes on ice to maintain a constant temperature between 8-10°C.

ii) **Iodination of histamine**

1 mCi ^{125}I-sodium (Amersham plc, UK) was mixed, using a pipette for 45 seconds, with 10 µl histamine solution (0.22 mg/ml in 0.5 molar phosphate buffer, pH8.0) and 10 µl chloramine-T solution (0.5 mg/ml in distilled water) in a conical glass tube prior to the addition of 10 µl sodium metabisulphite (30 mg/ml in distilled water) (reagents BDH, UK).

iii) **Conjugation**

120 µl of the 'activated steroid' was made up to 250 µl with dioxan and mixed with a pipette. 50 µl of the 'activated steroid' is taken and added to the ^{125}I-histamine, mixed prior to the addition of 10 µl of 0.1 molar sodium hydroxide and incubated on ice at 0°C for approximately 1 hour.

After incubation 900 µl of 0.1 molar hydrochloric acid and 1 ml ethyl ether were added to the mixture, vortexed and the organic and aqueous phases allowed to separate. The organic layer was

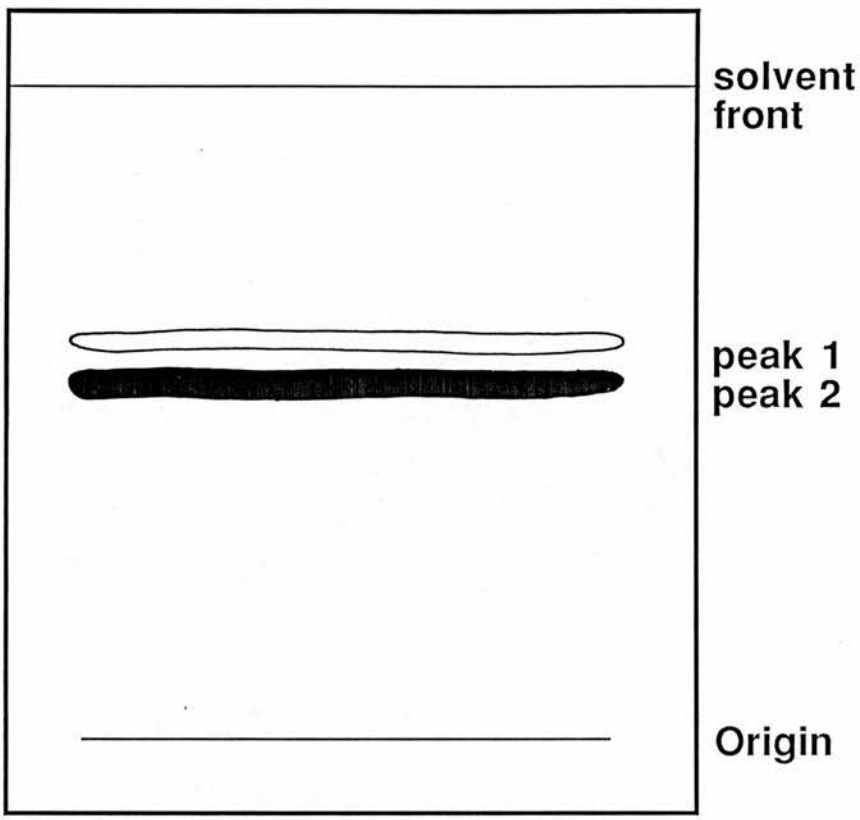


Figure 2.31: Thin layer chromatography of steroid-3-CMO-^{125}I-histamine - the active band is shaded.

aspirated and discarded into a suitable container for radioactive waste. 900 µl of 0.1 molar hydrochloric acid was added to the remaining aqueous layer, vortexed prior to the addition of 1 ml 0.5 molar phosphate buffer (pH 7.0) and 800 µl ethyl acetate and vortexed again. The organic and aqueous phases were allowed to separate over 10 minutes and the aqueous layer was aspirated and discarded into a suitable container for radioactive waste. The organic layer was further dried with the addition of anhydrous sodium sulphate (reagents BDH, UK).

The organic phase was spotted on to a TLC plate (200 x 200 mm aluminium precoated with 0.2 mm Silica gel 60, Merck, West Germany) and the products separated by thin layer chromatography using a mixture of chloroform: methanol: glacial acetic acid (90:10:1). The separation was run until the solvent front was just within the top of the plate, the plate dried in air and the radioactive bands detected by exposing the plate to X-ray film (NMB, Kodak-Pathe, France) for 30 minutes in a dark room. The film was developed and the appropriate band identified, removed and eluted overnight at 4°C in absolute alcohol. After TLC two bands of iodinated material are detectable and it is the lower band that contains the prepared tracer (Fig 2.32). Average yield was approximately 30 to 50%.

2.2.5.7: Preparation of dextran-coated charcoal

Dextran-coated charcoal was used for separation of androstenedione, testosterone and corticosterone assays and contained:

0.8 g gelatin (0.04% w/v) (Sigma Chemicals, USA)

1.2 g dextran T 70 (0.06% w/v) (Pharmacia Chemicals, Sweden)

12 g activated charcoal (0.6% w/v) (Sigma Chemicals, USA)

Gelatin was dissolved in heated 0.1 molar phosphate buffer, allowed to cool, mixed with dextran and activated charcoal, and made up to a final volume of 2 litres with 0.1 molar phosphate buffer.

2.2.5.8: Reproducibility characteristics of the multichannel superfusion-system

The coefficient of variation in flow rate (mean flow rate divided by standard deviation of the mean and expressed as percentage) was 1.5% (n = 10 columns) at 0.5 ml per minute. The between-column coefficient of variation in steroidogenesis (mean steroid concentration divided by the standard deviation of the mean and expressed as percentage) was obtained for a mixed population of adrenal and testicular cells by estimating each steroid concentration in the eluate from 10 parallel columns containing the same number of cells and treated with the same stimuli (ACTH over the dose range 1-100 pg/ml). This therefore takes into account error due to flow rate, loading the equivalent number of cells into each column and intra-assay precision. The respective values for progesterone, 17α-hydroxyprogesterone, androstenedione, testosterone and corticosterone are 25.1, 34.2, 32.4, 20.3 and 15.4%.

2.2.5.9: Statistics

To permit direct comparison of changes in steroid hormone

concentrations between experiments and between cell populations, responses were converted to the natural logarithm (ln) transforming data to a normal distribution (Feek, 1980) and analysed by Students' t-test. Data was processed by a BBC microcomputer (software, Dr C S Hetherington, Department of Clinical Chemistry, University of Newcastle-upon-Tyne).

Chapter 3

ADRENAL → GONAD INTERACTIONS *IN VIVO*

3.1: Introduction

The aim of these studies is to develop an *in vivo* model that demonstrates a possible interaction between the adrenal and the gonad in the male rat. The adrenal gland may secrete steroid precursors that can be used either directly by the testis in the secretion of testosterone or indirectly via the secretion of anterior pituitary LH. Previous studies (Kniewald, 1971; Kalra, 1977; Lescoat, 1982; Lescoat, 1984) have employed adrenalectomy as a model for the investigation of an interaction between the adrenal and testis. However, there are difficulties in the interpretation of these experiments.

i) In **acute studies** it is difficult to differentiate any effect that adrenalectomy may have upon plasma testosterone concentrations from the concomitant effect of surgical stress.

ii) Whilst **chronic studies** avoid this problem, it is difficult to differentiate any direct effect that adrenalectomy may have upon plasma testosterone concentrations from the debilitating effects of chronic glucocorticoid insufficiency. Furthermore, in any chronic study, resetting of anterior pituitary-gonadal relationships may mask the absence of any contribution made by the adrenal gland to testicular steroidogenesis.

Despite these reservations it has been shown that adrenalectomy prevents normal pubertal development (Lescoat, 1982), abolishes the circadian rhythm of testosterone secretion (Kalra, 1977) and reduces the stress-mediated rise in plasma testosterone concentrations when compared with sham-operated controls (Lescoat,

1984). In contrast, others have failed to demonstrate any effect of adrenalectomy on the plasma testosterone levels (McNeilly, 1980). Whilst results obtained from these experiments would appear to indicate that the adrenal gland may influence testicular steroidogenesis, they are inconsistent and difficult to interpret. The first set of experiments is designed to determine whether adrenalectomy does influence the testicular secretion of testosterone, and, if so, its mechanism.

3.2.1: The acute and chronic effects of adrenalectomy on plasma testosterone levels in the male rat

Adrenalectomy, sham adrenalectomy and gonadectomy were performed in male rats at 55 days of age under halothane anaesthesia. Postoperatively, adrenalectomised rats were maintained on saline 0.9% (w/v) ad libitum, whereas sham-operated and castrated animals were maintained on tap water. Blood was obtained for steroid assays by decapitation at the time intervals indicated (n = 3 animals per group at each time interval). Any changes in body weight, plasma corticosterone levels or plasma testosterone levels are illustrated in figures 3.1, 3.2 and 3.3 respectively.

i) Sham-adrenalectomised animals gained weight more rapidly than either castrated or adrenalectomised rats confirming previous studies (Lescoat, 1982). This presumably results from increased muscle protein breakdown and decreased protein synthesis caused by chronic glucocorticoid and testosterone insufficiency (Santidrian

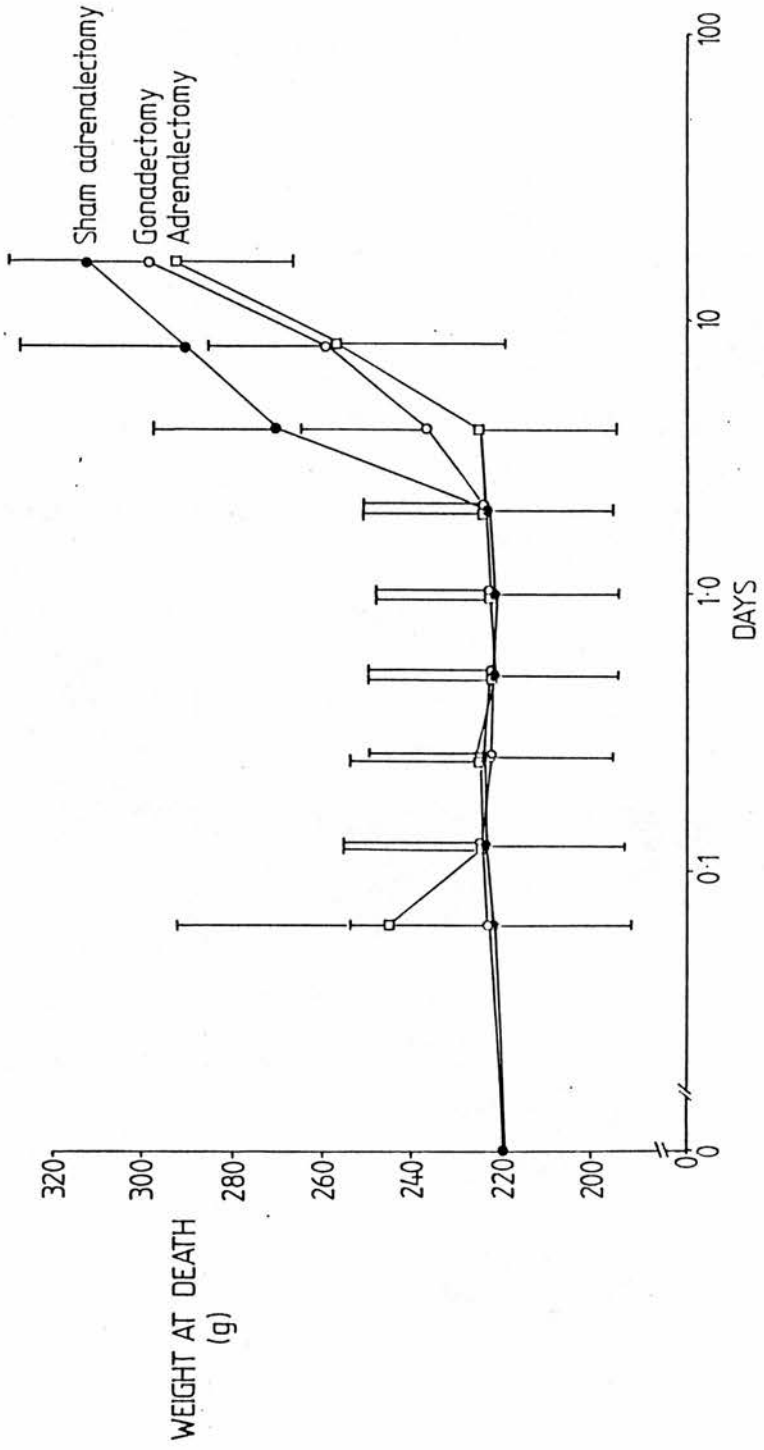


Figure 3.1: Mean ± SE body weight (g) at sacrifice following adrenalectomy, sham adrenalectomy or gonadectomy in intact male rats.

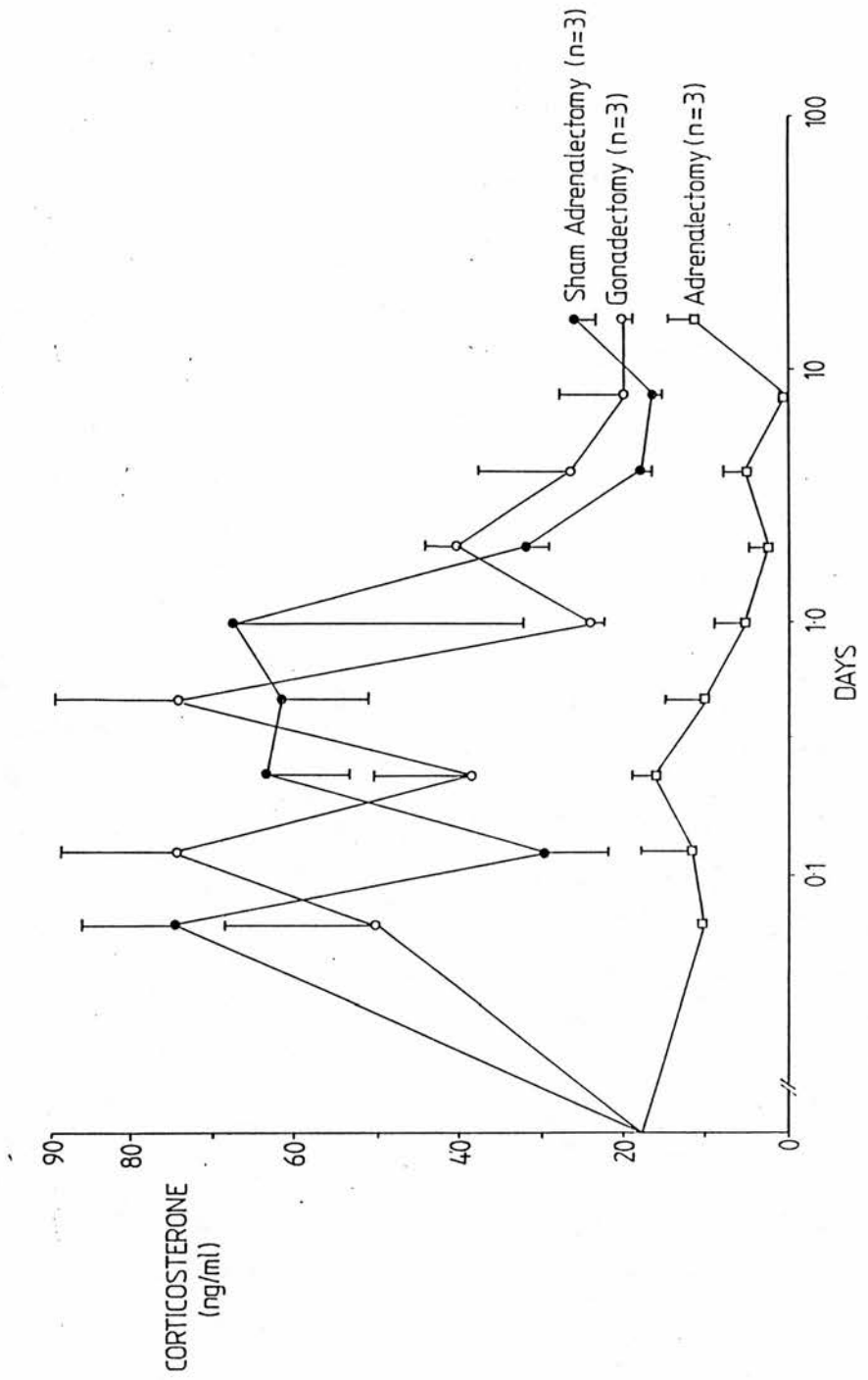


Figure 3.2: Mean ± SE plasma corticosterone levels (ng/ml) following adrenalectomy, sham adrenalectomy or gonadectomy in intact male rats.

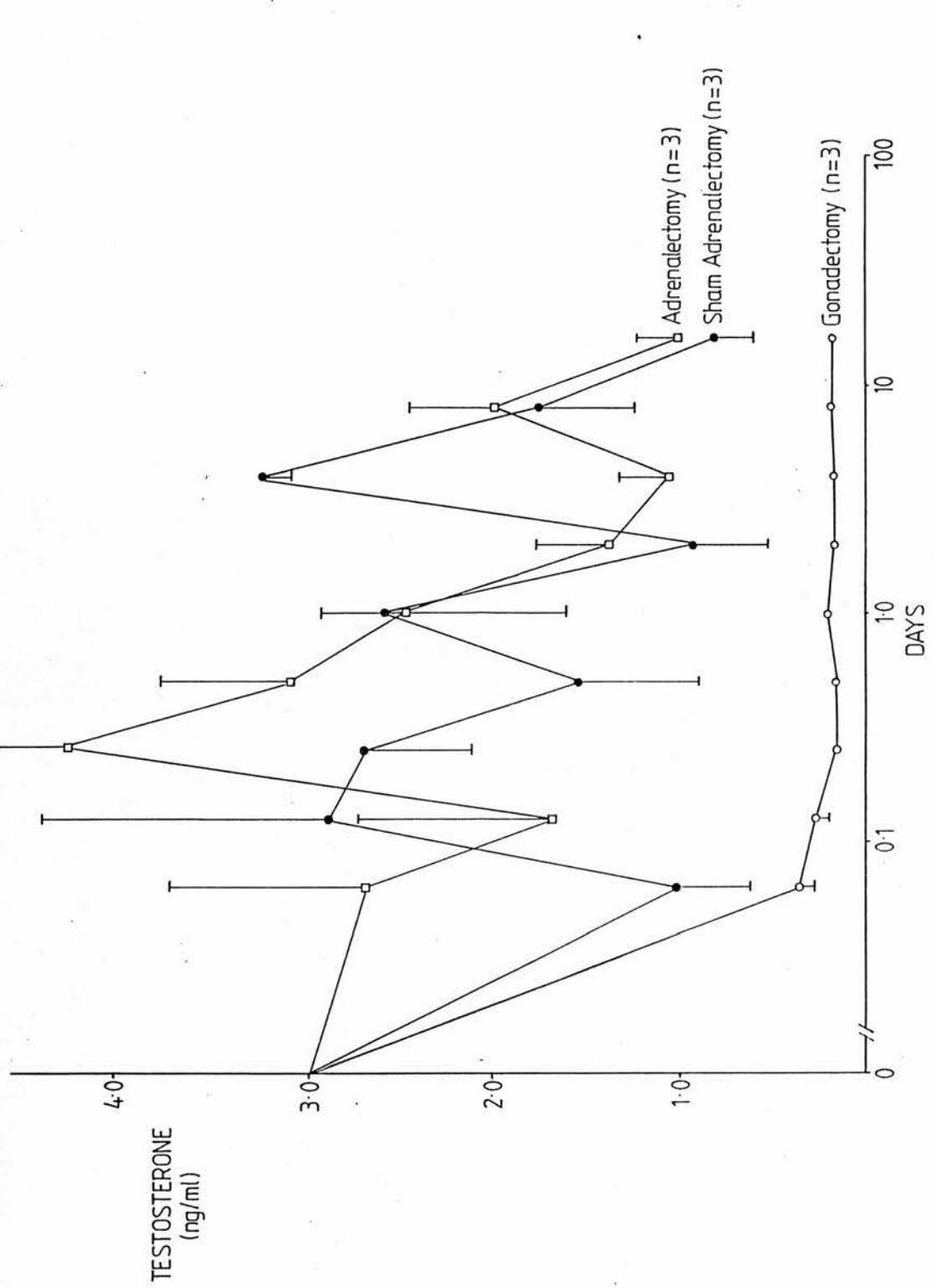


Figure 3.3: Mean ± SE plasma testosterone levels (ng/ml) following adrenalectomy, sham adrenalectomy or gonadectomy in intact male rats.

Alegre, 1980). If weight gain is any index of general well-being, the plasma testosterone response to adrenalectomy observed in previous studies (Kalra, 1977; Lescoat, 1982; Lescoat, 1984) may merely reflect the indirect effects of chronic adrenal insufficiency.

ii) Whilst there was a rise in plasma corticosterone levels following surgery in both sham-adrenalectomised and gonadectomised animals, there was no difference in the plasma corticosterone levels between the two groups. Bilateral adrenalectomy reduced plasma corticosterone concentrations as expected.

iii) No difference in plasma testosterone levels was observed between adrenalectomised and sham-adrenalectomised animals. Castration successfully reduced plasma testosterone concentrations as expected. This data conflicts with the results of previous studies (Kniewald, 1971; Lescoat, 1982). However, the small number of animals employed in the present study may explain the failure to achieve a significant difference in the plasma testosterone concentrations between the groups of animals. Unfortunately, small numbers of animals were necessary to permit accurate timing of the frequent samples that were generated in the early stages of the experiment.

For these reasons the experiment was extended to involve a larger number of animals, by assessing the plasma testosterone response to either adrenalectomy or sham adrenalectomy at 2 and 7 days following each procedure and compared with intact controls.

3.2.2: Plasma testosterone and corticosterone concentrations at 2 days following adrenalectomy and sham adrenalectomy in the male rat

Adrenalectomy and sham adrenalectomy were performed in 55-day-old male rats under halothane anaesthesia and compared with intact controls. Postoperatively, adrenalectomised rats were maintained on saline 0.9% (w/v) ad libitum whereas sham-operated and control animals were maintained on tap water. All experimental animals (n = 10 or 11 in each group) were left to recover from the effects of anaesthesia for 2 days before blood sampling by decapitation at 1100 h. Plasma corticosterone concentrations, plasma testosterone concentrations and body weight at death are illustrated in figure 3.4. Statistical significance of any changes in these parameters between the operated animals when compared with intact controls was assessed by Wilcoxon test.

i) Plasma corticosterone concentrations were significantly reduced in animals undergoing bilateral adrenalectomy (all levels undetectable, $p < 0.0005$) but not in animals undergoing sham operation (mean $\pm$ SE 157.5 $\pm$ 39.4 ng/ml, p not significant) when compared with unoperated controls (mean $\pm$ SE 121.9 $\pm$ 39.4 ng/ml).

ii) Plasma testosterone concentrations were significantly reduced in animals undergoing bilateral adrenalectomy (mean $\pm$ SE 1.16 $\pm$ 0.2 ng/ml, $p < 0.05$) and sham adrenalectomy (mean $\pm$ SE 1.14 $\pm$ 0.2 ng/ml, $p < 0.05$), when compared with intact controls (mean $\pm$ SE 2.56 $\pm$ 0.6 ng/ml). Hoewever, there was no difference in the plasma testosterone concentrations between adrenalectomised and sham-adrenalectomised animals.

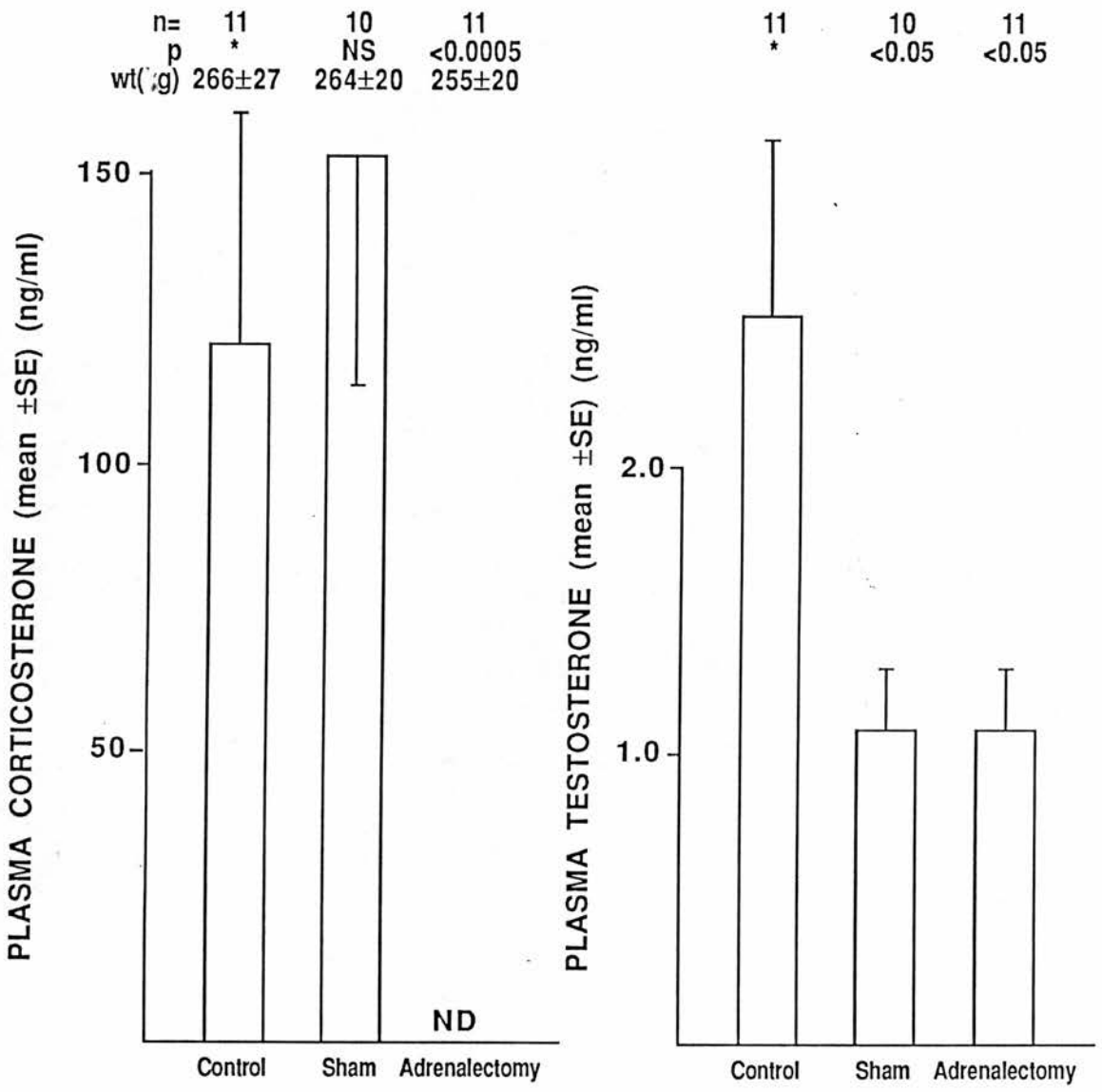


Figure 3.4: Mean ± SE plasma corticosterone levels (ng/ml), testosterone levels (ng/ml) and body weight (g) at 2 days following adrenalectomy and sham adrenalectomy in male rats when compared with intact controls.

iii) Body weights were comparable in each group.

These results are consistent with a non-specific effect of stress and general anaesthesia producing a depression of testicular testosterone secretion.

3.2.3: Plasma testosterone and corticosterone concentrations at 7 days following adrenalectomy and sham adrenalectomy in the male rat

Adrenalectomy and sham adrenalectomy were performed in 39-day-old male rats under halothane anaesthesia and compared with intact controls. Postoperatively, adrenalectomised rats are maintained on saline 0.9% (w/v) ad libitum. All experimental animals (n = 6 in each group) were left to recover from the effects of anaesthesia for 7 days before blood sampling by decapitation at 1100 h. Plasma corticosterone concentrations, plasma testosterone concentrations and body weight at death are illustrated in figure 3.5. Statistical significance of any changes in these parameters between the operated animals when compared with intact controls was assessed by Wilcoxon test.

i) Plasma corticosterone concentrations were significantly reduced in animals undergoing bilateral adrenalectomy (all levels undetectable, $p < 0.0005$) but not in animals undergoing sham operation (mean $\pm$ SE 336.2 $\pm$ 77.14 ng/ml, p not significant) when compared with intact controls (mean $\pm$ SE 295.7 $\pm$ 12.1 ng/ml).

ii) Plasma testosterone concentrations were not significantly reduced in animals undergoing bilateral adrenalectomy (mean $\pm$ SE 1.73 $\pm$ 0.3 ng/ml, p not significant) nor in sham-operated animals

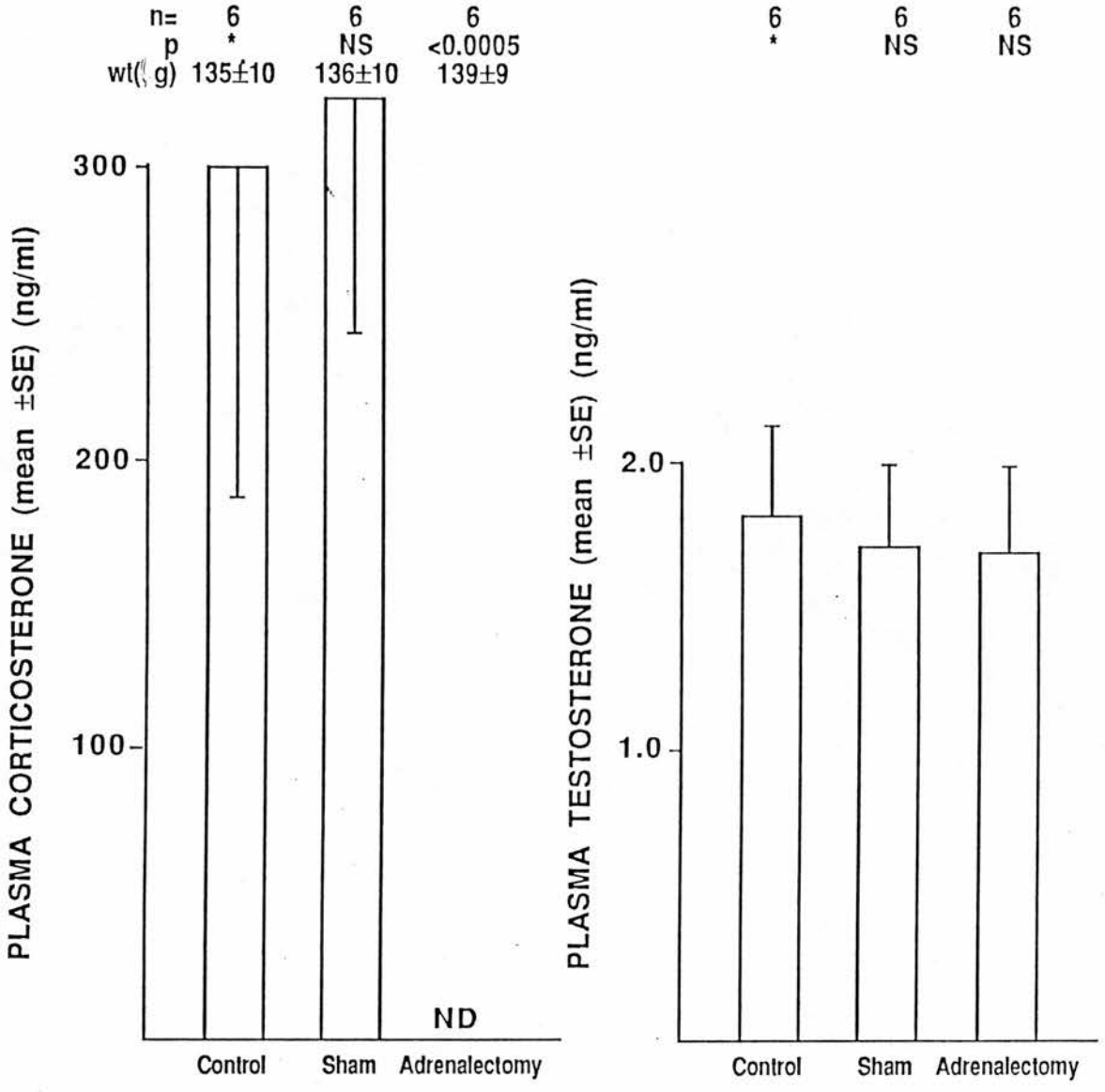


Figure 3.5: Mean ± SE plasma corticosterone levels (ng/ml), testosterone levels (ng/ml) and body weight (g) at 7 days following adrenalectomy and sham adrenalectomy in male rats when compared with intact controls.

(mean ± SE 1.75 ± 0.3 ng/ml, p not significant) when compared with intact controls (mean ± SE 1.8 ± 0.3 ng/ml).

iii) Body weights were comparable in each group.

These results are consistent with the amelioration of the non-specific effect of stress and general anaesthesia by testicular testosterone secretion.

In the male rat, during the **acute response to stress** (30-90 minutes), there is a rapid rise in the anterior pituitary secretion of LH, prolactin and ACTH that is accompanied by a progressive increase in the secretion of corticosterone by the adrenal gland and testosterone by the testis (Euker, 1975; Lorenzen, 1980). However, in the **chronic phase** (4-48 hours) of stress, there is a profound suppression of the pituitary-gonadal axis resulting in the suppression of the anterior pituitary secretion of LH and testicular secretion of testosterone (Free, 1973; Lorenzen, 1980; Childs, 1983; Ringstrom, 1984; Stahl, 1984). These changes may be mediated either by the direct action of stress on the hypothalamus and/or by the increased secretion of adrenal corticosteroids (Ringstrom, 1984; Mann, 1985). The latter is supported by the finding that the administration of pharmacological doses of glucocorticoid can achieve a similar inhibition of plasma LH and testosterone concentrations (Desjardins, 1971; Stahl, 1984; Ringstrom, 1984).

The administration of pharmacological doses of glucocorticoids to male rats was therefore used as a suitable model to study the influence of the adrenal gland on testicular steroidogenesis by

permitting suppression of the pituitary-adrenal axis as well as pituitary-gonadal axis. Subsequent stimulation of the suppressed adrenal gland with ACTH will determine whether the adrenal can provide steroid precursors that can either be utilised by the testis in the secretion of testosterone directly or indirectly by stimulation of anterior pituitary gonadotrophs.

3.2.4: The effect of dexamethasone suppression on plasma testosterone levels in the male rat

Adequate suppression of the pituitary-gonadal axis has been previously achieved with pharmacological doses of glucocorticoid employing dexamethasone 10-1000 μg daily for 5 days (Saez, 1977; Bambino, 1981; Stahl, 1984), corticosterone 3.0 mg daily for 5 days and cortisol 100 mg by implantation for a 4 day period (Ringstrom, 1984). The use of dexamethasone has the advantage of being a synthetic glucocorticoid with little cross-reactivity with the assays employed in this study. Preliminary experiments (data not shown) have shown that an oral regimen of dexamethasone 100 μg at 0900, 1700 and 2300 h on the day before the study and again at 0900 h on the day of study achieves adequate suppresssion of both plasma corticosterone and testosterone levels.

Dexamethasone was administered to male rats aged 39 days old (n = 6) and plasma corticosterone and testosterone levels are compared with untreated controls (n = 6). Blood for steroid hormone analysis was obtained by decapitation at 1100 h. Plasma corticosterone concentrations, plasma testosterone concentrations

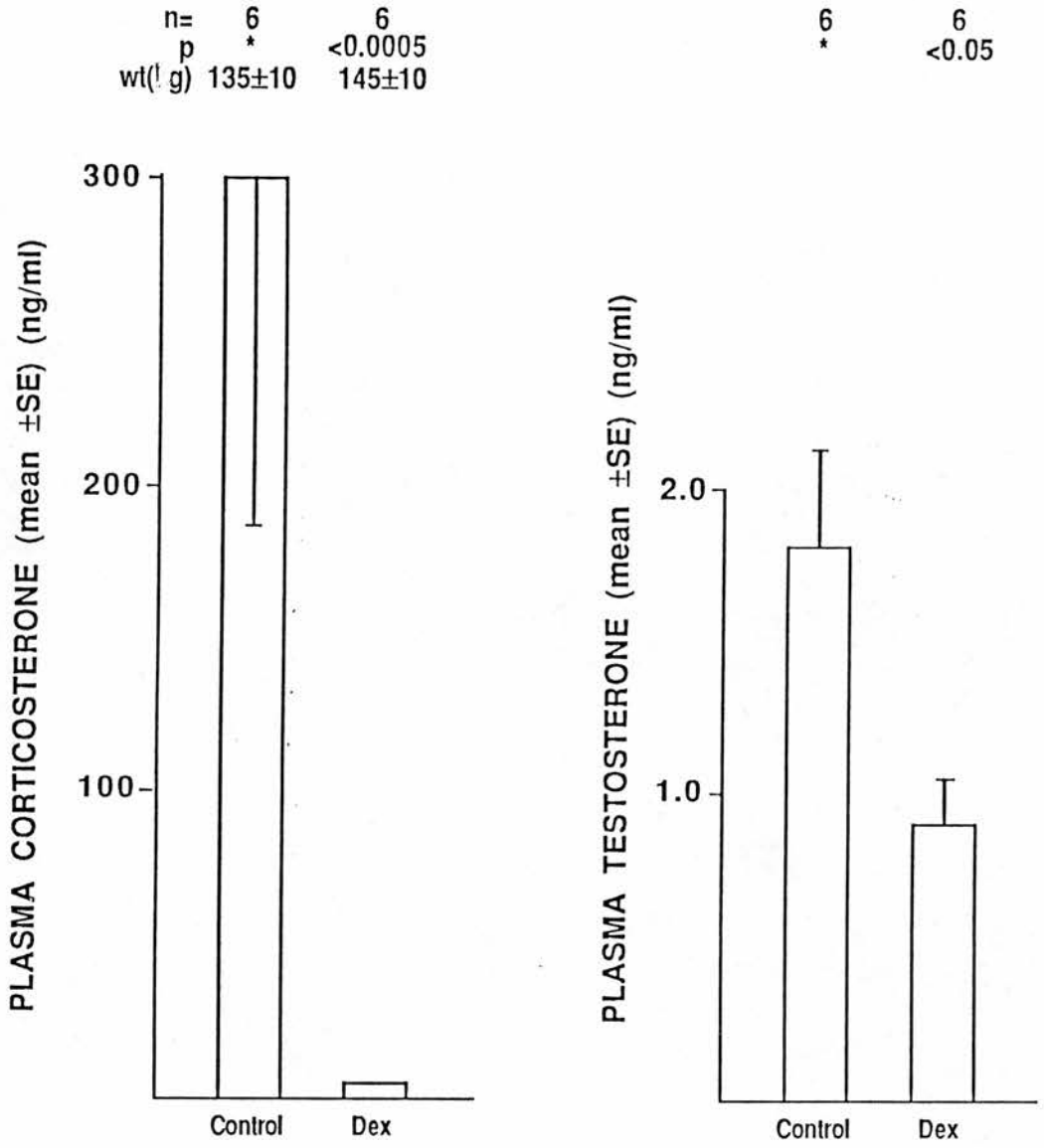


Figure 3.6: The effect of dexamethasone suppression on plasma corticosterone (ng/ml), plasma testosterone levels (ng/ml) and body weight (g) on male rats when compared with untreated controls.

and body weight at death are illustrated in figure 3.6. Statistical significance of any changes in these parameters between treated animals when compared with untreated controls was assessed by Wilcoxon test.

i) Plasma corticosterone concentrations were significantly reduced in animals treated with dexamethasone (mean ± SE 5.7 ± 2.4 ng/ml, $p < 0.0005$) compared with untreated controls (mean ± SE 295.7 ± 12.1 ng/ml).

ii) Plasma testosterone concentrations were significantly reduced in animals treated with dexamethasone suppression (mean ± SE 0.92 ± 0.2 ng/ml, $p < 0.05$) when compared with untreated controls (mean ± SE 1.8 ± 0.3 ng/ml).

iii) Body weights were comparable in each group.

These results demonstrate suppression of both pituitary-adrenal and pituitary-gonadal axes with pharmacological doses of glucocorticoid. Dexamethasone was then administered to castrated male rats in order to assess whether its action is mediated by suppression of the anterior pituitary secretion of LH or by direct effects upon the testis.

3.2.5: The effect of dexamethasone suppression on the anterior pituitary secretion of LH in castrated male rats

In order to determine the effect of pharmacological doses of glucocorticoid on anterior pituitary LH secretion, male rats of 55 days of age were castrated under halothane anaesthesia and allowed to recover for 7 days prior to the oral administration of either

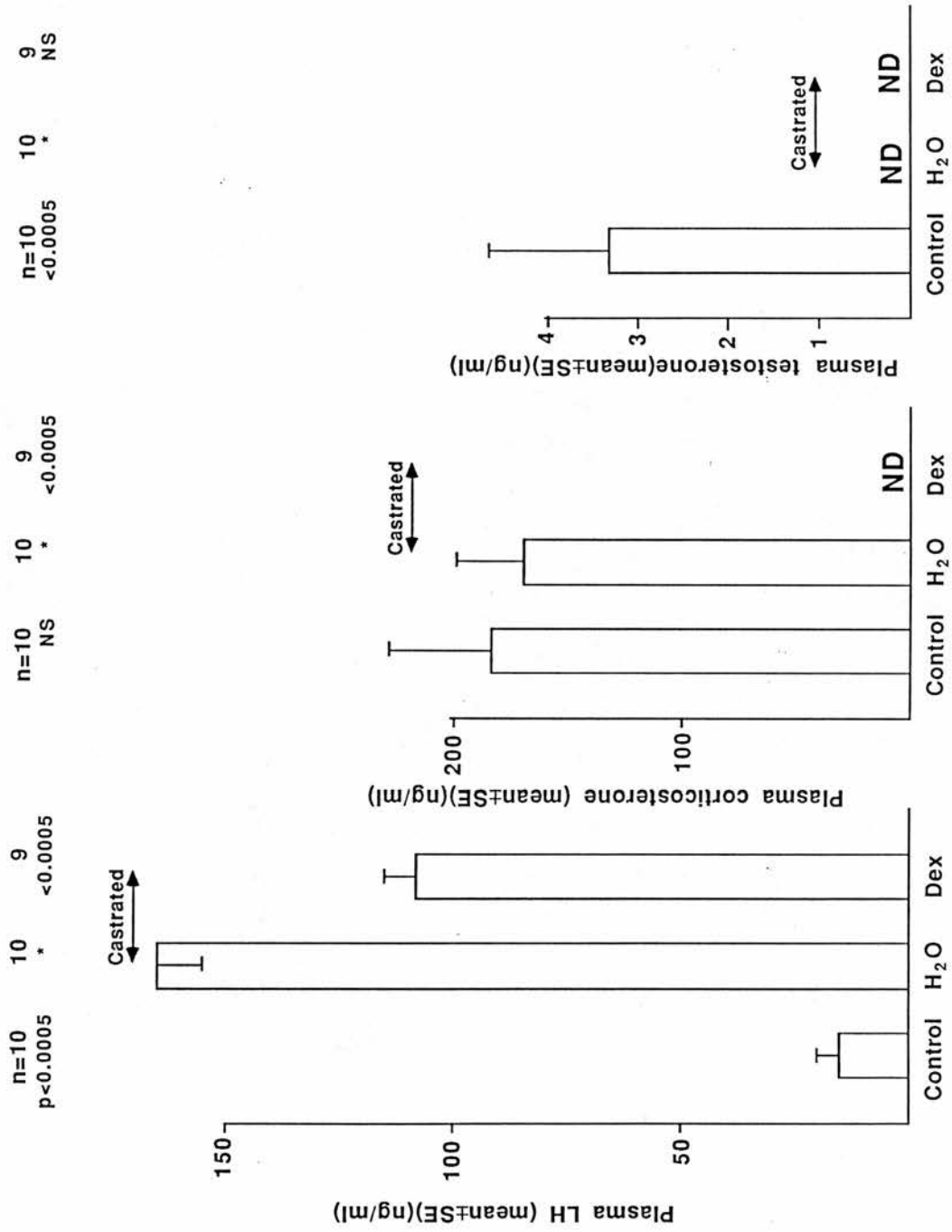


Figure 3.7: Plasma LH, corticosterone and testosterone levels in castrated, untreated male rats when compared with dexamethasone-suppressed, castrated animals and untreated, intact controls.

water or dexamethasone 100 μg at 0900, 1700 and 2300 h on the day before the study and again at 0900 h on the day of study and compared with intact and untreated controls. Blood for steroid hormone analysis was obtained by decapitation at 1100 h. Statistical significance of any changes in plasma corticosterone, testosterone and LH concentrations between untreated, castrated animals (n = 10) when compared with dexamethasone-treated, castrated animals (n = 9) and untreated intact, controls (n = 10) was assessed by Wilcoxon test. Results are illustrated in figure 3.7.

i) There was a significant elevation of plasma LH concentrations following castration in untreated animals (mean ± SE 165.9 ± 27.0 ng/ml) when compared with untreated, intact controls (mean ± SE 15.1 ± 14.7 ng/ml, $p < 0.0005$). However, the rise in plasma LH levels in response to castration was significantly reduced in dexamethasone-treated, animals (mean ± SE 97.0 ± 37.3 ng/ml, $p < 0.0005$) when compared with untreated animals.

ii) Castration reduces testosterone levels to below the level of detection in both groups when compared with intact controls (mean ± SE 3.6 ± 0.9 ng/ml, $p < 0.0005$).

iii) The administration of dexamethasone to castrated animals reduces plasma corticosterone levels (undetectable, $p < 0.0005$) when compared with their untreated castrated counterparts (mean ± SE 173 ± 32 ng/ml) and untreated, intact controls (mean ± SE 185.2 ± 38 ng/ml).

This experiment confirms previous studies demonstrating

suppression of the pituitary-gonadal axis in male rats with pharmacological doses of glucocorticoid (Smith, 1971; Baldwin, 1974; Baldwin, 1979; Stahl, 1984; Ringstrom, 1984).

3.2.6 The effect of ACTH over the dose range 0.01-10 μg on intact, dexamethasone-suppressed male rats when compared with intact, untreated controls

In order to determine the optimal dose of intraperitoneal ACTH necessary to achieve plasma corticosterone and testosterone concentrations in dexamethasone-treated animals comparable with untreated controls, either water (untreated controls, n = 10) or dexamethasone 100 μg at 0900, 1700 and 2300 h was administered orally on the day before the study and again at 0900 h on the day of study, and saline (n = 10) or ACTH was administered intraperitoneally over the dose range 0.1-10 μg (n = 10 in each group) to dexamethasone-suppressed male rats aged 50 days at 1000 h and blood collected for steroid hormone analysis by decapitation at 1100 h. Preliminary studies (data not shown) showed an increase in both plasma corticosterone and testosterone concentrations at 60 minutes following the intraperitoneal injection of ACTH but the concentrations of these plasma steroids declined shortly thereafter. Plasma corticosterone concentrations, plasma testosterone concentrations, plasma progesterone concentrations and body weight at death in response to ACTH over the dose range are illustrated in figure 3.8. Statistical significance of any changes in any of these parameters in treated animals when compared with untreated

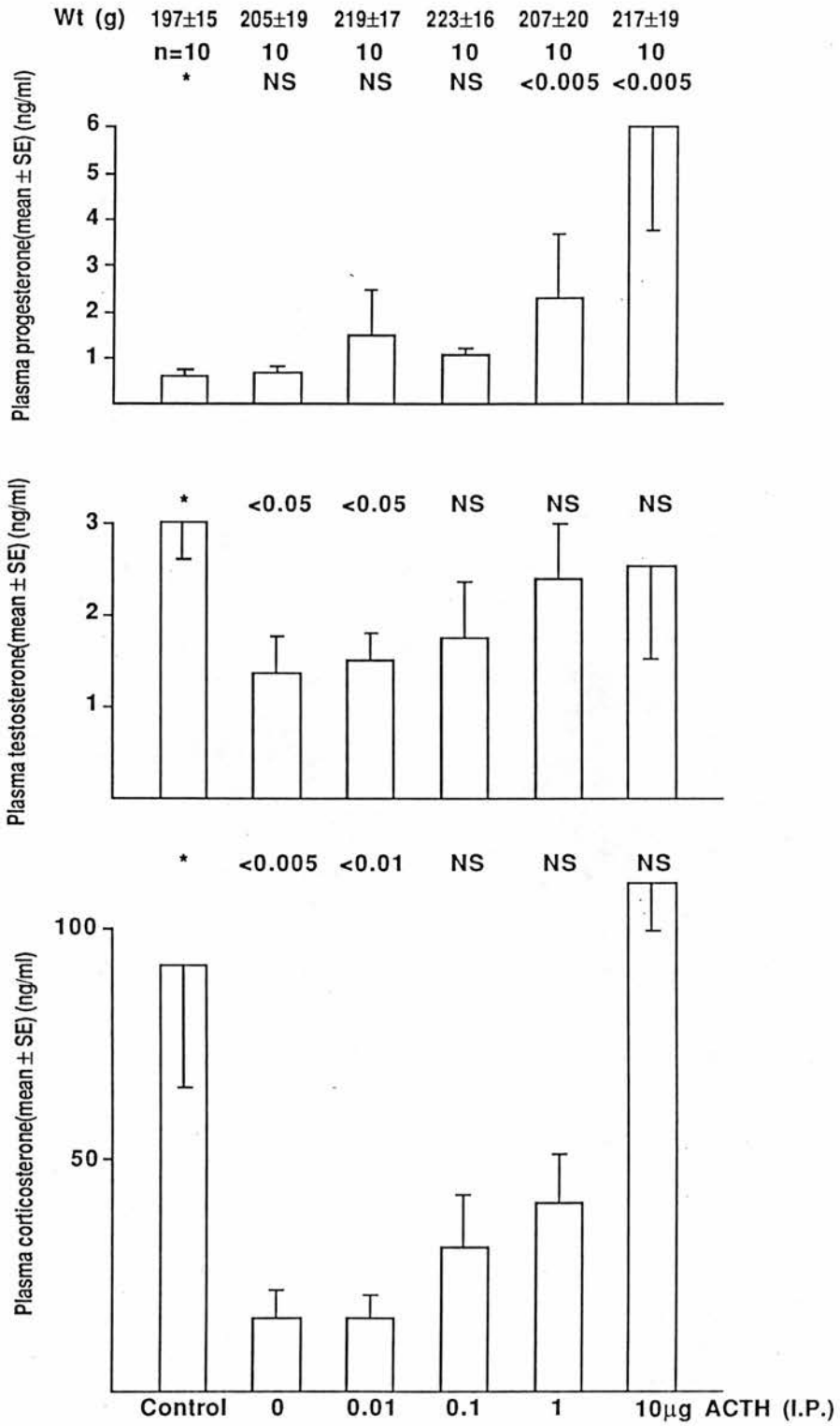


Figure 3.8: The effect of ACTH in dexamethasone-suppressed, intact male rats on plasma corticosterone (ng/ml), plasma testosterone levels (ng/ml), plasma progesterone levels (ng/ml) and body weight (g) when compared with untreated, intact controls.

controls was assessed by Wilcoxon test.

i) Plasma corticosterone concentrations were significantly reduced in animals treated with dexamethasone alone (mean ± SE 17.0 ± 5.0 ng/ml, $p < 0.005$), in animals treated with dexamethasone and ACTH 0.01 μg (mean ± SE 16.9 ± 3.2 ng/ml, $p < 0.01$) but not with animals treated with dexamethasone and ACTH 0.1 μg (mean ± SE 30.8 ± 10.2 ng/ml, p not significant), ACTH 1.0 μg (mean ± SE 38.5 ± 11.3 ng/ml, p not significant) and ACTH 10 μg mean ± SE 110.2 ± 60 ng/ml, p not significant) when compared with untreated, intact controls (mean ± SE 93.3 ± 26.6 ng/ml).

ii) Plasma testosterone concentrations were significantly reduced in animals treated with dexamethasone alone (mean ± SE 1.4 ± 0.3 ng/ml, $p < 0.05$), in animals treated with dexamethasone and ACTH 0.01 μg (mean ± SE 1.5 ± 0.4 ng/ml, $p < 0.05$) but not with animals treated with dexamethasone and ACTH 0.1 μg (mean ± SE 1.8 ± 0.4 ng/ml, p not significant), ACTH 1.0 μg (mean ± SE 2.4 ± 0.7 ng/ml, p not significant) and ACTH 10 μg/ml (mean ± SE 2.6 ± 0.9 ng/ml, p not significant) when compared with untreated controls (mean ± SE 3.1 ± 0.7 ng/ml).

iii) Plasma progesterone concentrations were not significantly different in animals treated with dexamethasone alone (mean ± SE 0.5 ± 0.16 ng/ml, p not significant), and ACTH 0.01 μg (mean ± SE 0.5 ± 0.07 ng/ml, p not significant), in animals treated with dexamethasone and ACTH 0.1 μg (mean ± SE 1.5 ± 1.1 ng/ml, p not significant), but increased in animals treated with dexamethasone and ACTH 1.0 μg (mean ± SE 2.3 ± 1.4 ng/ml, $p < 0.05$)

and ACTH 10 μg/ml (mean ± SE 6.2 ± 2.2 ng/ml, $p < 0.05$) when compared with untreated, intact controls (mean ± SE 0.5 ± 0.16 ng/ml).

Thus the administration of ACTH 10 μg to dexamethasone-suppressed rats is able to restore plasma corticosterone and plasma testosterone to levels similar to those found in untreated controls, whereas plasma progesterone exceeds those found in untreated controls, although well within the normal range of 1-25 ng/ml described by other authors (Lorenzen, 1980). There may be, therefore, some dissociation of adrenal steroidogenesis in response to ACTH by the dexamethasone-suppressed adrenal. Nevertheless, restoration of plasma testosterone concentrations by the administration of ACTH implies that an adrenal product may increase plasma testosterone levels.

In order to demonstrate that the source of increased secretion of testosterone in response to ACTH is due to the adrenal secretion of a product that is capable of stimulating testicular steroidogenesis, the plasma testosterone response to ACTH was studied in the following groups:

i) dexamethasone-suppressed, castrated rats

ii) dexamethasone-suppressed, adrenalectomised rats

iii) dexamethasone-suppressed, intact rats

and compared with untreated, intact rats.

3.2.7: The plasma progesterone, corticosterone, testosterone and LH responses in castrated, dexamethasone-suppressed male rats treated with and without ACTH (10 μg) when compared with intact, untreated controls

In order to determine whether the adrenal gland secretes significant amounts of testosterone, the effect of dexamethasone suppression (100 μg 8 hourly for 24 hours) upon plasma testosterone levels in castrated animals, with (n = 14) and without (n = 15) ACTH stimulation (10 μg intraperitoneally), was compared with those of intact, untreated controls (n = 15). Data pooled from three separate experiments is illustrated in figure 3.9 and statistical significance of any changes of the parameters was assessed by Wilcoxon test.

i) The administration of dexamethasone to castrated rats significantly depressed both plasma corticosterone (mean ± SE 7.5 ± 2.3 ng/ml) and progesterone (mean ± SE 0.63 ± 0.05 ng/ml) levels when compared with untreated, intact controls (mean ± SE plasma corticosterone 222 ± 18.5 ng/ml, $p < 0.001$ and mean ± SE plasma progesterone 5.8 ± 1.6 ng/ml, $p < 0.001$), whereas the administration of dexamethasone plus ACTH to castrated rats significantly increased both plasma corticosterone (mean ± SE 117.6 ± 20.4 ng/ml, $p < 0.001$) and plasma progesterone levels (mean ± SE 2.5 ± 0.09 ng/ml, $p < 0.001$) when compared with castration and dexamethasone alone.

ii) In castrated, dexamethasone-suppressed rats, plasma testosterone levels are undetectable and plasma LH levels were

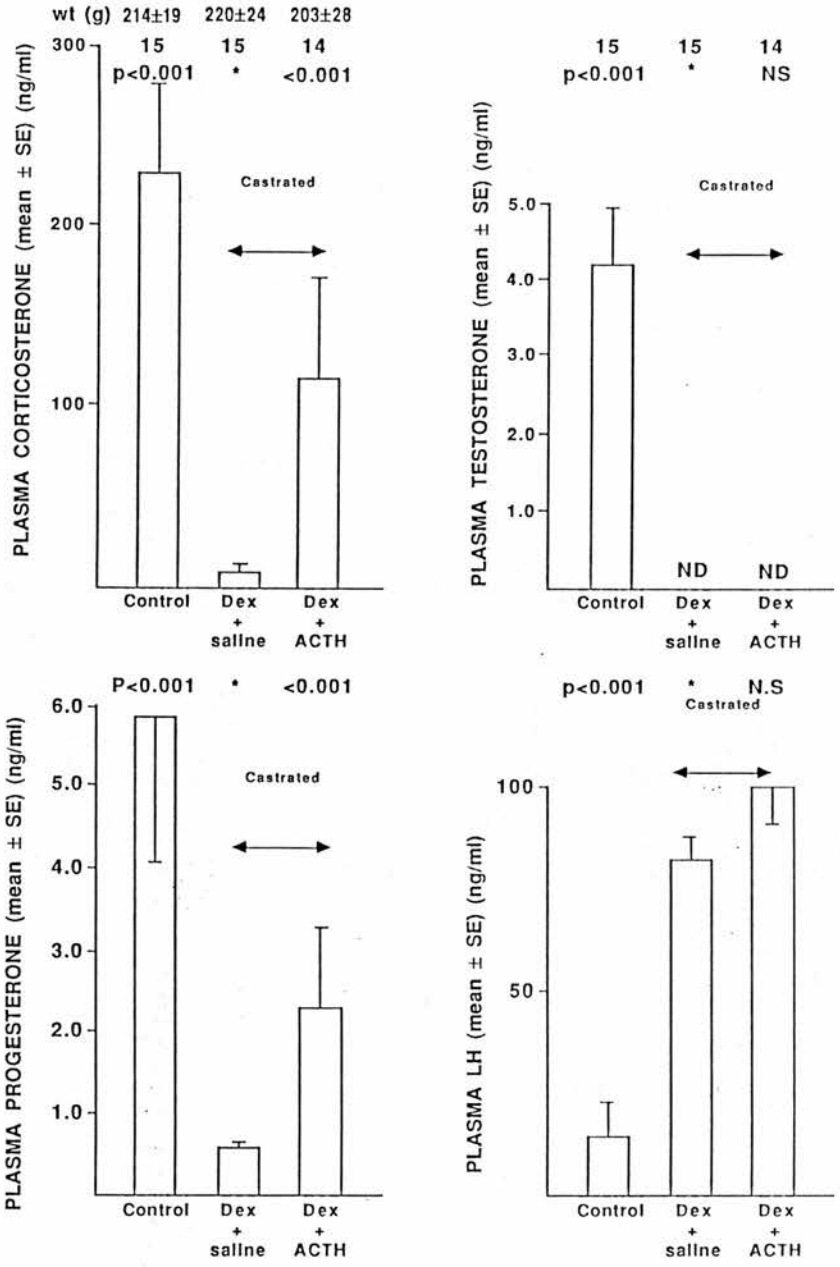


Figure 3.9: Mean ± SE body weight (g), plasma corticosterone (ng/ml), plasma progesterone (ng/ml), plasma testosterone (ng/ml) and plasma LH (ng/ml) concentrations in castrated rats treated with dexamethasone when compared to castrated animals, treated with dexamethasone and ACTH, and compared with intact, untreated controls.

significantly elevated (mean ± SE plasma LH 82.6 ± 5.9 ng/ml) when compared with untreated, intact controls (mean ± SE plasma testosterone 4.44 ± 2.7 ng/ml, p <0.001 and mean ± SE plasma LH 13.1 ± 2.4 ng/ml, p <0.001) but both plasma testosterone (undetectable, p not significant) and plasma LH concentrations (mean ± SE 101.6 ± 7.0 ng/ml, p not significant) failed to change in castrated dexamethasone-suppressed rats following the administration of ACTH when compared with castration and dexamethasone alone.

iii) Body weights were not significantly different in the three groups.

Whilst castration lowers plasma testosterone levels and elevates plasma LH levels as expected, ACTH stimulation of dexamethasone-suppressed castrated animals increases the adrenal secretion of corticosterone and progesterone but **the adrenal gland does not secrete significant amounts of testosterone in vivo.**

3.2.8: The plasma progesterone, corticosterone, testosterone and LH responses in adrenalectomised, dexamethasone-suppressed male rats treated with and without ACTH (10 μg) when compared with intact, untreated controls

In order to determine whether pharmacological doses of glucocorticoid inhibit and whether ACTH directly stimulates testicular steroidogenesis, the effect of dexamethasone suppression (100 μg 8 hourly for 24 hours) upon plasma testosterone and LH levels in adrenalectomised animals, with (n = 12) and without (n = 12) ACTH stimulation (10 μg intraperitoneally), was compared with intact,

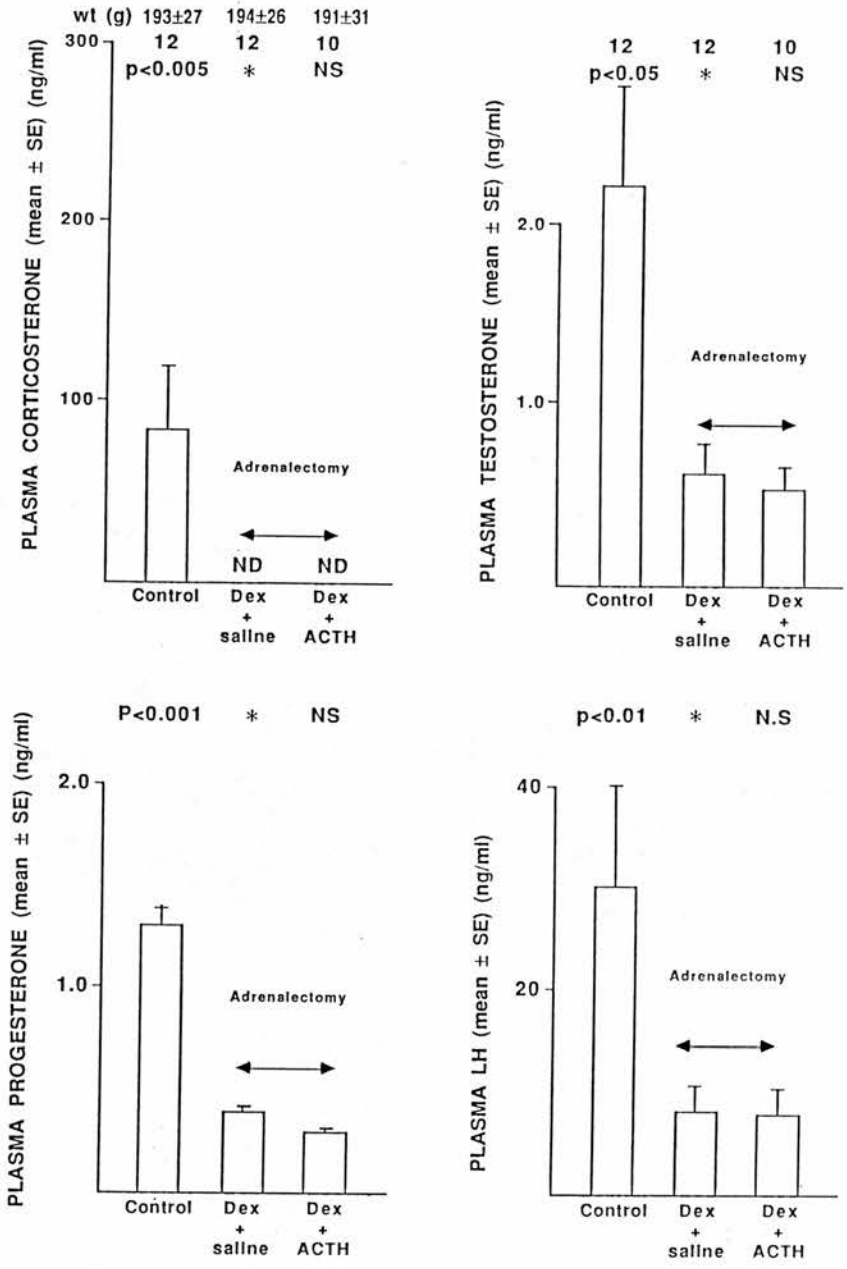


Figure 3.10: Mean ± SE body weight (g), plasma corticosterone (ng/ml), plasma progesterone (ng/ml), plasma testosterone (ng/ml) and plasma LH (ng/ml) concentrations in adrenalectomised rats treated with dexamethasone when compared to adrenalectomised animals treated with dexamethasone and ACTH (10 μg, ip) and intact, untreated controls.

untreated controls (n = 12). Data pooled from three separate experiments is illustrated in figure 3.10 and statistical significance of any changes in the parameters was assessed by Wilcoxon test.

i) In adrenalectomised rats dexamethasone suppression significantly reduced both plasma testosterone (mean ± SE 0.6 ± 0.15 ng/ml, $p < 0.05$) and plasma LH levels (mean ± SE 6.4 ± 0.2 ng/ml) when compared with untreated, intact controls (mean ± SE plasma testosterone 2.2 ± 0.6 ng/ml, $p < 0.05$ and mean ± SE plasma LH 29.6 ± 5.2 ng/ml, $p < 0.01$).

Thus, **pharmacological doses of glucocorticoid inhibit testicular steroidogenesis by suppressing the pituitary-gonadal axis in the male rat in vivo.**

ii) The administration of ACTH to adrenalectomised, dexamethasone-suppressed rats failed to reverse the fall in either plasma corticosterone (undetectable, $p < 0.0005$), plasma progesterone (mean ± SE 0.3 ± 0.02 ng/ml, p not significant) or plasma testosterone levels (mean ± SE 0.5 ± 0.12 ng/ml, p not significant) when compared with adrenalectomised animals treated with dexamethasone alone (plasma corticosterone undetectable, mean ± SE plasma progesterone 0.4 ± 0.03 ng/ml and mean ± SE plasma testosterone 0.6 ± 0.15 ng/ml).

Thus, **ACTH does not stimulate testicular steroidogenesis directly in the male rat in vivo.**

iii) Body weights were not significantly different in the three groups.

3.2.9: The plasma progesterone, corticosterone, testosterone and LH responses in intact, dexamethasone-suppressed rats treated with and without ACTH (10 μg) when compared with intact, untreated controls

In order to determine whether the adrenal gland may contribute either directly or indirectly to testicular steroidogenesis, the effect of dexamethasone suppression (100 μg 8 hourly for 24 hours) upon plasma testosterone and LH levels in intact animals, with (n = 27) and without (n = 27) ACTH (10 μg intraperitoneally), was compared with intact, untreated controls (n = 27). Data pooled from three separate experiments is illustrated in figure 3.11 and statistical significance of any changes in the parameters was assessed by Wilcoxon test.

i) In intact animals, the administration of dexamethasone significantly reduced both plasma corticosterone (mean ± SE 14.0 ± 3.0 ng/ml), plasma testosterone (mean ± SE 1.6 ± 0.3 ng/ml) and plasma LH (mean ± SE 8.0 ± 0.8 ng/ml) but not plasma progesterone levels (mean ± SE 0.64 ± 0.09 ng/ml) when compared with untreated controls (mean ± SE plasma corticosterone 88.5 ± 16.3 ng/ml, $p < 0.001$, mean ± SE plasma testosterone 3.7 ± 0.7 ng/ml, $p < 0.001$, mean ± SE plasma LH 17.2 ± 2.8 ng/ml and mean ± SE plasma progesterone 0.72 ± 0.09 ng/ml, p not significant).

ii) In contrast, the administration of ACTH to intact, dexamethasone-suppressed animals significantly increased plasma corticosterone (mean ± SE 168.8 ± 29.4 ng/ml, $p < 0.001$) plasma progesterone (mean ± SE 1.93 ± 0.25 ng/ml, $p < 0.001$) and plasma

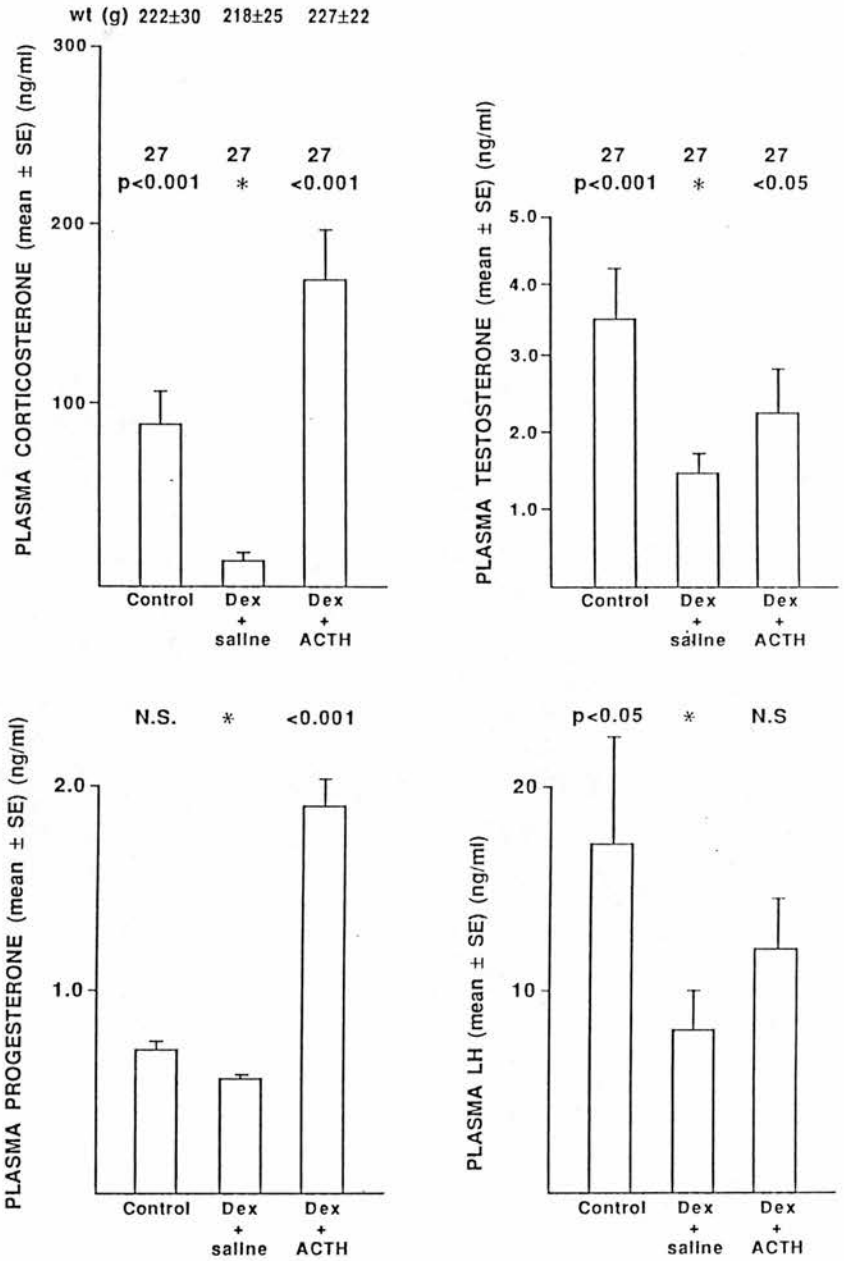


Figure 3.11: Mean ± SE body weight (g), plasma corticosterone (ng/ml), plasma progesterone (ng/ml), plasma testosterone (ng/ml) and plasma LH (ng/ml) concentrations in intact rats treated with dexamethasone when compared with intact animals treated with dexamethasone and ACTH (10 µg, ip) and intact, untreated controls.

testosterone (mean 2.4 ± 0.6 ng/ml, $p < 0.05$) but not plasma LH levels (mean ± SE 15.1 ± 1.1 ng/ml, p not significant) when compared with intact animals treated with dexamethasone alone.

iii) Body weights were not significantly different in the three groups.

Thus, **the adrenal gland contributes to testicular steroidogenesis in the rat** in vivo. Since increased testosterone secretion in response to ACTH is not associated with a significant change in plasma LH levels it would appear that the adrenal stimulation of the testis is mediated by direct means rather than indirectly by means of the pituitary-gonadal axis.

It is clear from this in vivo model that there is some evidence to support the concept that the adrenal gland may secrete steroid precursors that can stimulate the testicular secretion of testosterone. Parallel in vitro studies have implicated a role for progesterone as agents directly in the mediation of adrenal ➟ gonadal interactions (see section 4). Progesterone was therefore administered to dexamethasone-suppressed rats, in doses to achieve physiological plasma progesterone concentrations, in order to determine whether progesterone can acutely stimulate the testicular secretion of testosterone.

3.2.10 The effect of the administration of progesterone (100 µg, sc) or saline on plasma corticosterone, plasma progesterone and plasma testosterone levels in intact, dexamethasone-suppressed animals when compared with intact, untreated controls

In initial studies, the administration of progesterone to dexamethasone-suppressed animals in doses up to 100 μg by the intraperitoneal route failed to achieve any significant elevation of plasma progesterone concentrations (all levels undetectable, data not shown) and consequently failed to achieve a change in plasma testosterone levels (data not shown). Presumably either the absorption of progesterone by this route is inadequate or presystemic metabolism by the liver is such to prevent any significant quantities of progesterone from entering the circulation. In subsequent experiments, progesterone was administered by the subcutaneous route and preliminary experiments (data not shown) demonstrate that a subcutaneous injection of 100 μg achieves plasma progesterone concentrations that are within the physiological range.

In three separate experiments, either water (untreated, intact controls) or dexamethasone 100 μg at 0900, 1700 and 2300 h was administered orally on the day before the study and again at 0900 h on the day of study and either progesterone (100 μg) or saline was administered subcutaneously to dexamethasone-suppressed, intact male rats aged 50 days at 1000 h and blood collected for steroid hormone analysis by decapitation at 1100 h. Statistical significance of any changes in parameters in pooled data between intact animals treated with dexamethasone alone (n = 39) when compared with intact animals treated with dexamethasone and progesterone (n = 39) and untreated, intact controls (n = 39) was assessed by Wilcoxon test and the results are illustrated in figure

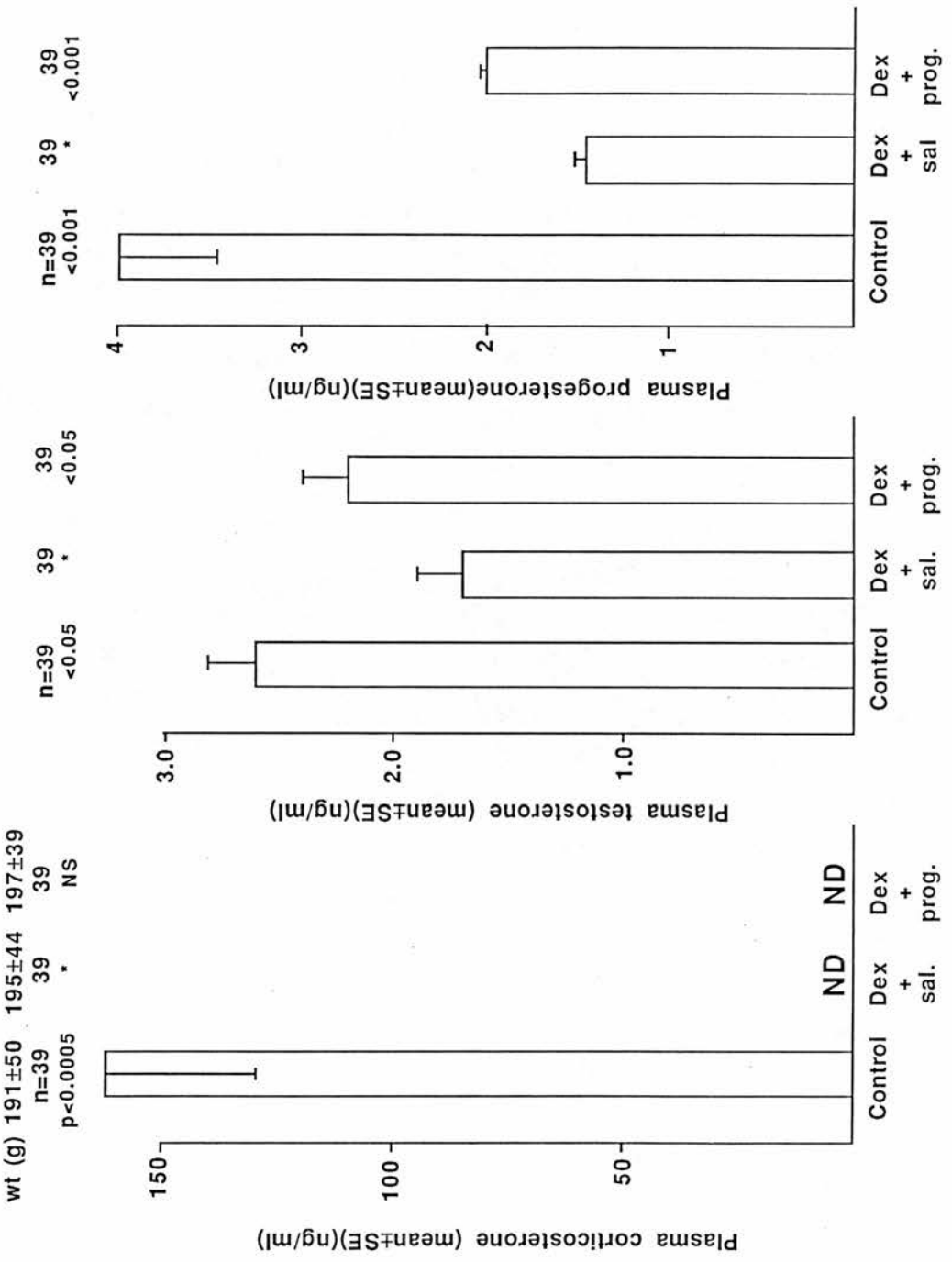


Figure 3.12: Plasma corticosterone, plasma progesterone and plasma testosterone levels in intact rats treated with dexamethasone when compared with intact animals treated with dexamethasone and progesterone intact, untreated controls.

3.12.

i) In intact animals, the administration of dexamethasone significantly reduced both plasma corticosterone (not detectable), plasma testosterone (mean ± SE 1.7 ± 0.2 ng/ml) and plasma progesterone (mean ± SE 1.4 ± 0.01 ng/ml) levels when compared with intact, untreated controls (mean ± SE plasma corticosterone 164.4 ± 26.9 ng/ml, $p < 0.001$, mean ± SE plasma testosterone 2.6 ± 0.2 ng/ml, $p < 0.05$, and mean ± SE plasma progesterone 3.9 ± 0.6 ng/ml, $p < 0.001$).

ii) In contrast, the administration of progesterone to intact, dexamethasone-suppressed animals significantly increased plasma progesterone (mean ± SE 2.1 ± 0.1 ng/ml, $p < 0.001$) and plasma testosterone (mean 2.2 ± 0.2 ng/ml, $p < 0.05$) but not plasma corticosterone (not detectable, p not significant) levels when compared with intact animals treated with dexamethasone alone.

iii) Body weights were not significantly different in the three groups.

It is concluded that the administration of progesterone to dexamethasone-suppressed male rats in doses achieving physiological plasma progesterone concentrations stimulates the testicular secretion of testosterone.

3.3: Discussion

This series of experiments illustrates the successful development of an *in vivo* model of adrenal ➟ gonadal interactions in the male rat. The action of adrenal steroids upon testicular

steroidogenesis is complex; whilst the adrenal secretion of glucocorticoid indirectly inhibits the testicular secretion of testosterone (by suppressing the anterior pituitary secretion of LH), the adrenal secretion of progesterone directly stimulates the testicular secretion of testosterone.

These studies confirm previous results in which the administration of progesterone (10 mg in oil by injection) to either intact, adrenalectomised or hypohpysectomised but untreated male rats increased plasma testosterone concentrations at 6-8 hours (Kalra, 1980). In both intact and adrenalectomised animals this effect was mediated without altering plasma gonadotrophin levels. In other studies the administration of progesterone to male rats, either intact or castrated (with or without the prior administration of testosterone), demonstrated little influence on the anterior pituitary secretion of LH (Brown-Grant, 1974; Aiyer, 1976; Fink, 1977). Thus the ability of progesterone to stimulate testicular steroidogenesis appears be mediated directly rather than indirectly by stimulation of the anterior pituitary secretion of LH.

The ability of the testis to metabolise exogenous progesterone varies drastically during the sexual development and maturation of the male rat. The addition of physiological concentrations of progesterone to fetal testes in vitro stimulates testosterone production (Habert, 1986). Testicular tissue of newborn rats in vitro (1-10 days old) is able to convert 25-60% of progesterone to androgens (principally testosterone) but this ability is lost (less

than 1% conversion) as the testes mature over the period 10-40 days of age, only to return again in the fully mature animal (40% conversion) (Steinberger, 1968). If the adrenal secretion of progesterone is capable of stimulating testicular steroidogenesis and the ability of the testis to respond to progesterone is limited to certain periods of testicular maturation, these observations would tend to suggest that the adrenal gland may have an important role in the sexual development of the male rat. In male rats both plasma progesterone and testosterone exhibit circadian rhythms that are related to the photoperiod and adrenalectomy subsequently abolishes these circadian rhythms (Kalra, 1977). Thus the adrenal secretion of progesterone may exert profound effects on the daily secretion of testosterone by the testis.

In the **acute phase of stress** (the first 90 minutes) there is a rise in plasma LH and plasma testosterone concentrations which can be ameliorated by prior adrenalectomy (Lescoat, 1984), suggesting that the rise in plasma testosterone concentrations is, in part, explained by the stress-related secretion of adrenal precursors that stimulate testicular steroidogenesis. However, during the **chronic phase of stress** (4 to 48 hours), plasma LH and testosterone levels fall due to non-specific suppression of LH secretion that may be partly explained by increased secretion of glucocorticoids by the adrenal as well as inhibition of the hypothalamic-pituitary-gonadal axis.

3.4: Conclusions

The significance of these *in vivo* findings is to provide evidence in the male rat that:

i) the adrenal gland does not secrete significant amounts of androgens or testosterone.

ii) the major part of androgen production is derived from the testis.

iii) the administration of pharmacological doses of glucocorticoid can suppress the hypothalamic-pituitary-testicular axis.

iv) the adrenal can directly stimulate testicular steroidogenesis, and this is probably mediated by the secretion of adrenal steroid precursors such as progesterone.

Chapter 4

ADRENAL → GONAD INTERACTIONS *IN VITRO*

4.1: Introduction

The aim of these experiments is to develop an *in vitro* system that illustrates a possible interaction between the adrenal and the testis in the rat. Studies on isolated whole perfused testis have provided much information on the biosynthetic pathways of the testis (Chubb, 1979a; Chubb, 1979b) and studies involving the isolated whole perfused adrenal have added to our knowledge of adrenal steroidogenesis (Sibley, 1981). However, the preparation of isolated cells permits a more readily accessible method of studying possible hormonal interactions between the adrenal and the testis.

Static incubation systems have been successfully used to demonstrate the synthesis and secretion of hormones from endocrine cells *in vitro*. Whilst this approach has the advantage of simplicity, it does not necessarily provide an ideal method for the investigation of cellular function (see sections 1.1.4 and 1.2.4).

In contrast, the superfusion system is a dynamic model for hormone action. This system has been well described as a model for adrenal steroidogenesis (Lowry, 1974) as well as testicular steroidogenesis (Davies, 1979; Wu, 1985) and has previously been used succesfully to study interactions between isolated cells derived from the same organ (Bartlett, 1985). Whilst the juxtaposition of adrenal and testicular cells *in vitro* does not necessarily reflect the tight anatomical and functional arrangement of cells that exists *in vivo* it does provide an opportunity to explore of a direct interaction between the steroid-secreting cells

isolated from the adrenal and testis.

4.1.1: Superfusion of isolated testicular cells with eluate from stimulated adrenal cells

In one experiment, isolated adrenal cells (1 x 10^6 per column, n = 2) were stimulated with ACTH (100 pg/ml) and the eluate was then superfused through columns of isolated testicular cells (10 x 10^6 per column, n = 2). Following stimulation of adrenal cells with ACTH there was an increase in corticosterone secretion as expected, but superfusion of the eluate through the isolated testicular cells stimulated testosterone secretion (Fig 4.1), supporting the concept that adrenal cells may contribute a product that can be utilised by testicular cells for the purposes of steroidogenesis. The basis of this preliminary experiment was therefore used for subsequent experiments as a model for adrenal ➔ gonad interactions. In these experiments adrenal and testicular cells were mixed within each column for technical convenience, to enable individual experiments to be 'scaled-up' to employ 10 columns.

4.1.2: Response of a mixture of isolated adrenal and testicular cells to ACTH over the dose range 1-100 pg/ml

Steroid responses to the administration of ACTH over the dose range 1-100 pg/ml from a typical experiment are illustrated in figures 4.2 to 4.6. The experiment used 10 parallel columns: six columns consisted of a mixture of isolated adrenal (1 x 10^6 per column) and isolated testicular cells (10 x 10^6 per column),

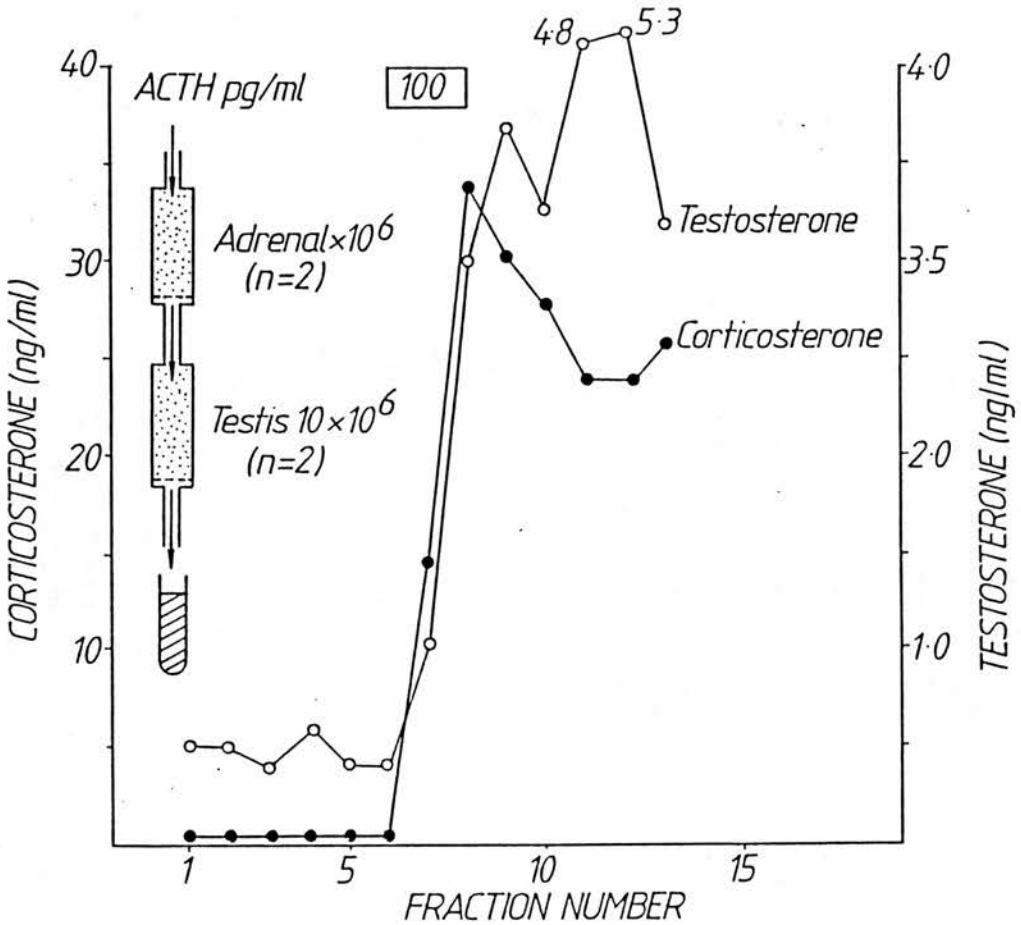


Figure 4.1: Mean testosterone (O——O) and corticosterone (●——●) responses to ACTH 100 pg/ml following the superfusion of eluate from isolated superfused adrenal cells through isolated superfused testicular cells (n = 2 columns). Each fraction represents 5 minutes.

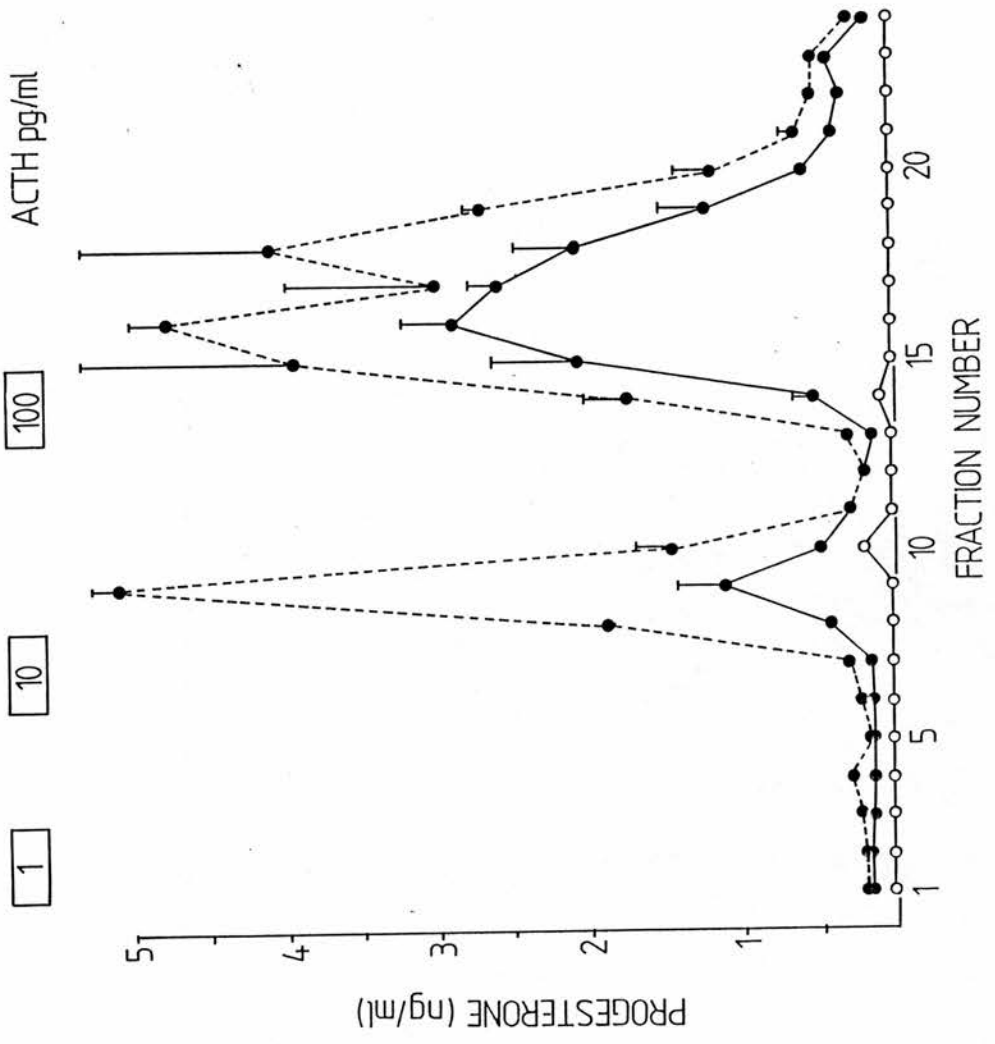


Figure 4.2: Mean ± SE progesterone response to ACTH 1-100 pg/ml by isolated superfused adrenal cells (●----●), testicular cells (○——○) and a mixed cell population (●——●). Each fraction represents 5 minutes.

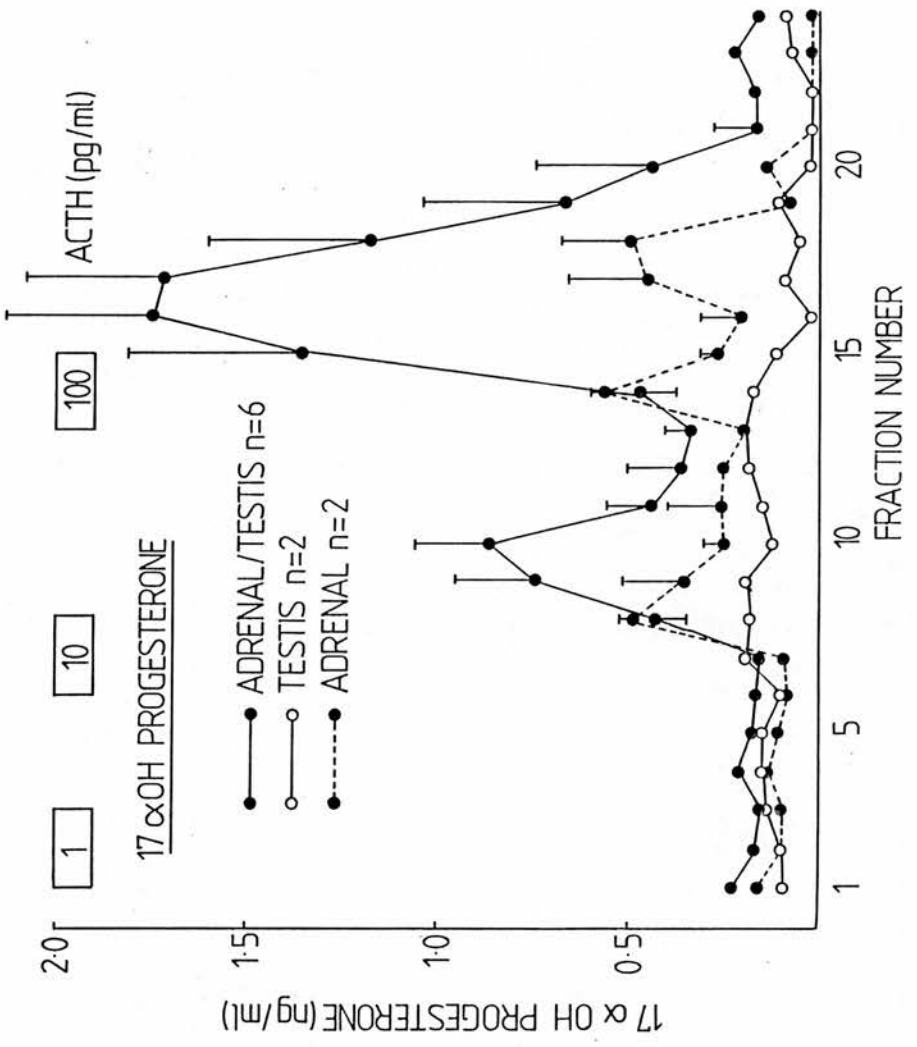


Figure 4.3: Mean ± SE 17α-hydroxyprogesterone responses to ACTH 1-100 pg/ml by isolated superfused adrenal cells (●----●), testicular cells (○——○) and a mixed cell population (●——●). Each fraction represents 5 minutes.

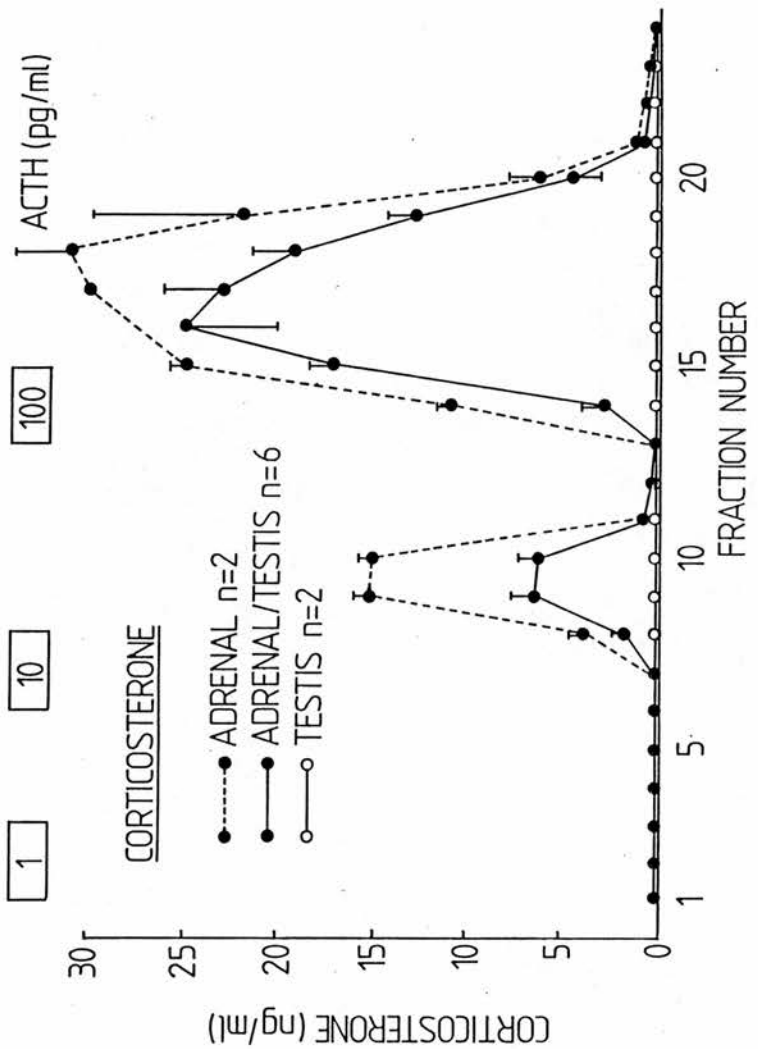


Figure 4.4: Mean ± SE corticosterone responses to ACTH 1-100 pg/ml by isolated superfused adrenal cells (●----●), testicular cells (○——○) and a mixed cell population (●——●). Each fraction represents 5 minutes.

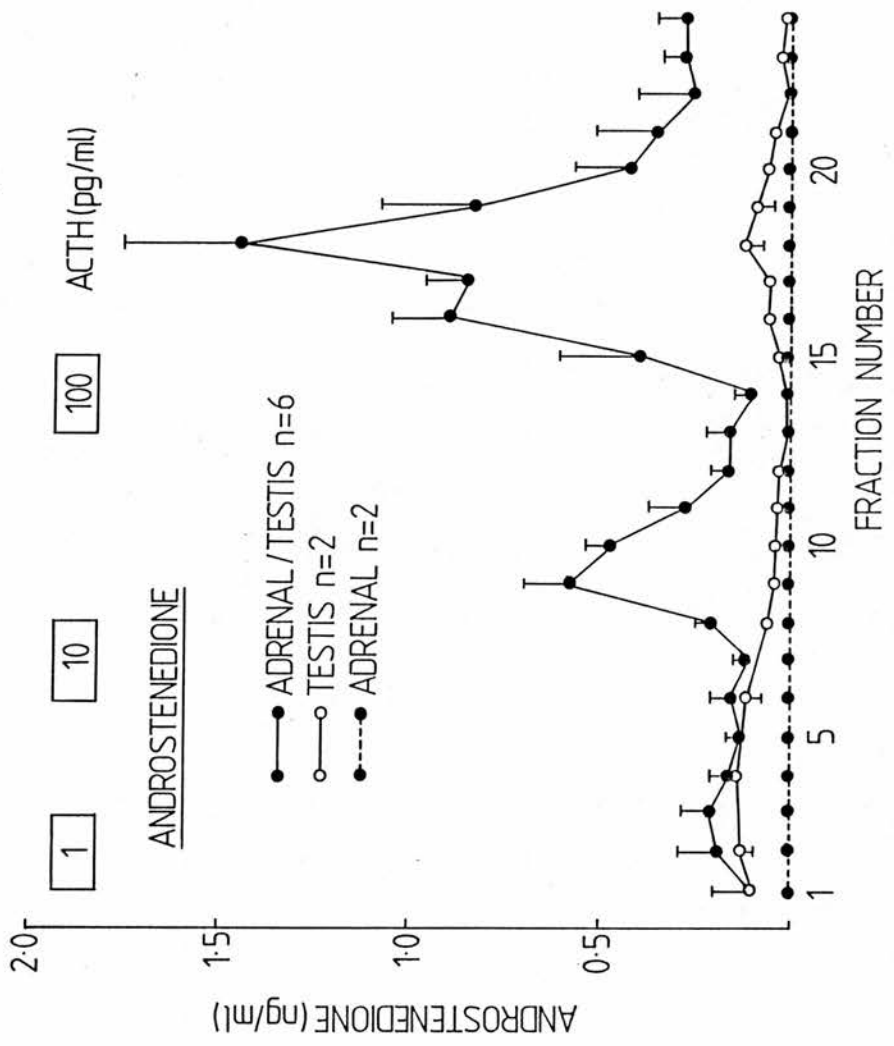


Figure 4.5: Mean ± SE androstenedione responses to ACTH 1-100 pg/ml by isolated adrenal cells (●----●), testicular cells (○——○) and a mixed cell population (●——●). Each fraction represents 5 minutes.

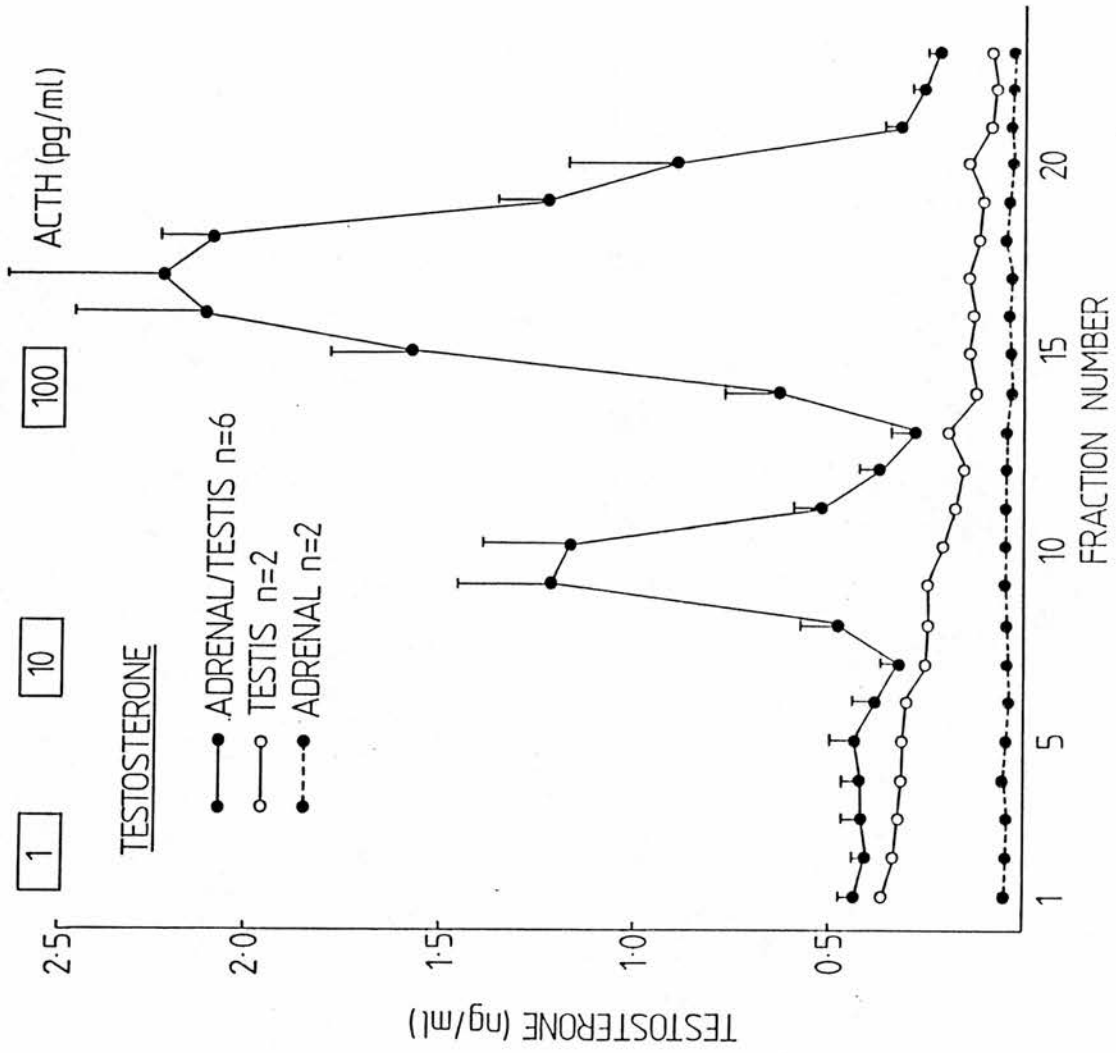


Figure 4.6: Mean ± SE testosterone responses to ACTH 1-100 pg/ml by isolated superfused adrenal cells (●----●), testicular cells (○——○) and a mixed cell population (●----●). Each fraction represents 5 minutes.

two columns of isolated adrenal cells alone (1 x 10^6 cells per column) and two columns of isolated testicular cells alone (10 x 10^6 cells per column). Over the dose range employed there was a dose-dependent secretion of progesterone (Fig 4.2), 17α-hydroxyprogesterone (Fig 4.3) and corticosterone (Fig 4.4) from isolated adrenal cells, with or without the juxtaposition of testicular cells, whereas secretion of these hormones by testicular cells alone was negligible. In addition there was a dose-dependent secretion of androstenedione (Fig 4.5) and testosterone (Fig 4.6) by the mixture of isolated adrenal and testicular cells that was not seen when the two cell types were separate. Furthermore, the juxtaposition of testicular with adrenal cells decreased the concentrations of progesterone and corticosterone and increased the concentrations of 17α-hydroxyprogesterone.

4.1.3: Pooled data of isolated cell response to ACTH 100 pg/ml

The steroid responses to the administration of ACTH 100 pg/ml were pooled from five consecutive experiments and analysed by transforming the data to the natural logarithm (ln) for the purpose of statistical analysis by Student's t-test. The results are illustrated in figures 4.7 to 4.11 (note the logarithmic scale). The concentrations of 17α-hydroxyprogesterone, androstenedione and testosterone were significantly increased and the concentrations of progesterone and corticosterone significantly reduced in the eluate of the mixed cell population (n = 6 columns per experiment) when compared with that of the adrenal cells alone (n = 2 columns per

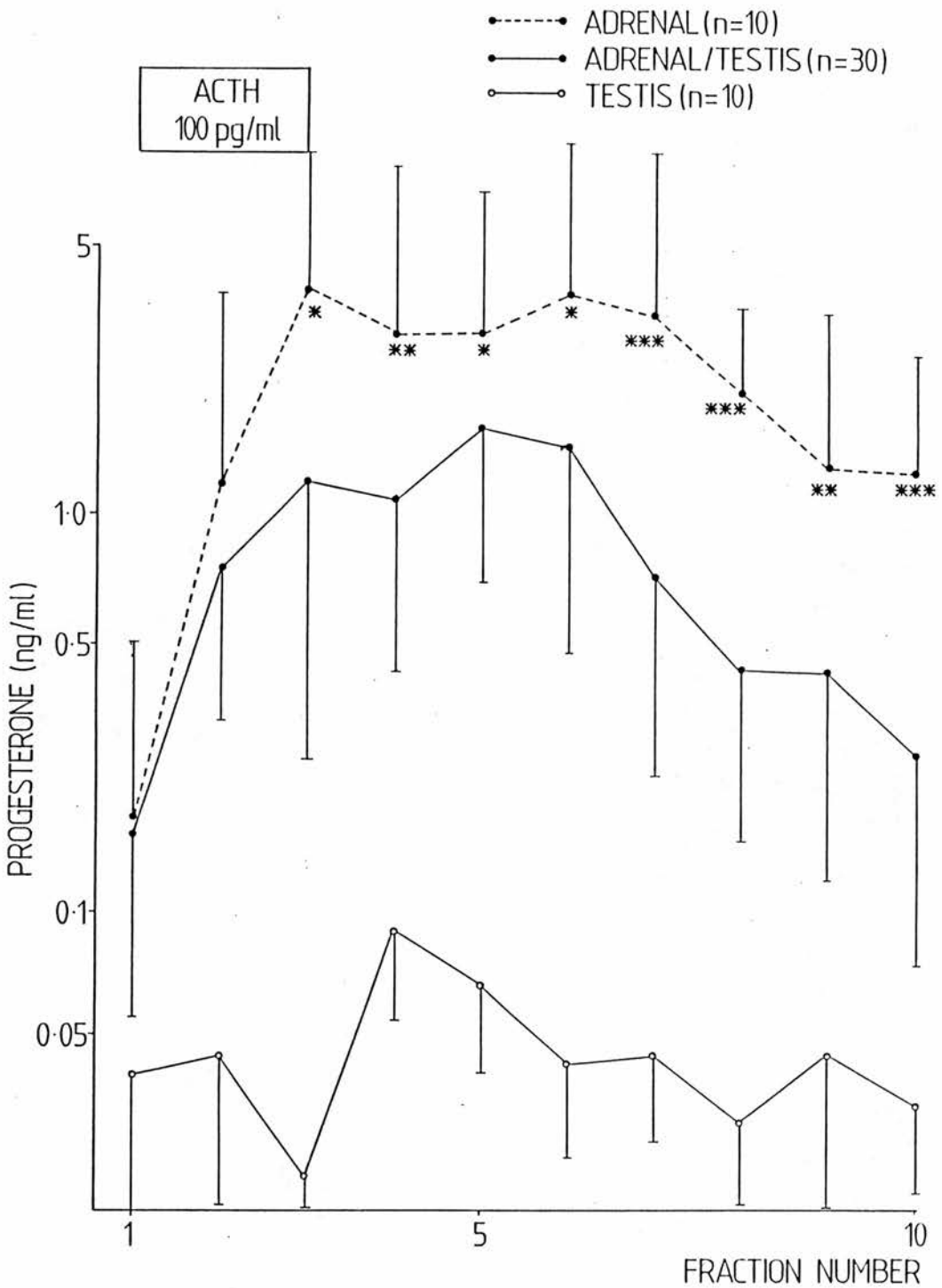


Figure 4.7: Pooled data from 5 separate experiments illustrating the progesterone response to ACTH 100 pg/ml by isolated superfused adrenal cells (●----●), testicular cells (O——O) and a mixed cell population (●——●). (* = $p < 0.05$, ** = $p < 0.005$ and *** = $p < 0.0005$).

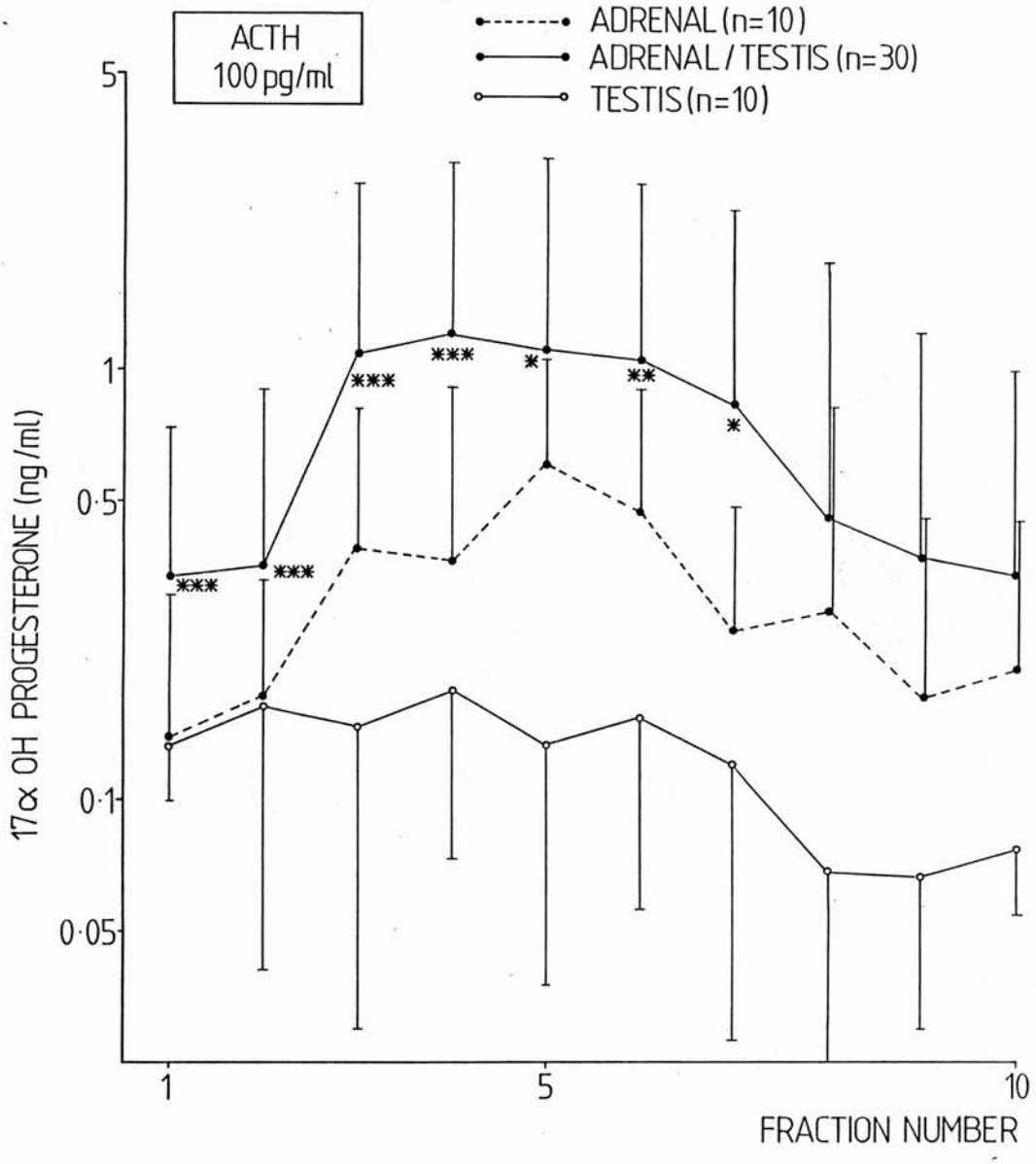


Figure 4.8: Pooled data from 5 separate experiments illustrating the 17α-hydroxyprogesterone response to ACTH 100 pg/ml by isolated adrenal cells (●----●), testicular cells (O——O) and a mixed cell population (●——●). (* = $p < 0.05$, ** = $p < 0.005$ and *** = $p < 0.0005$).

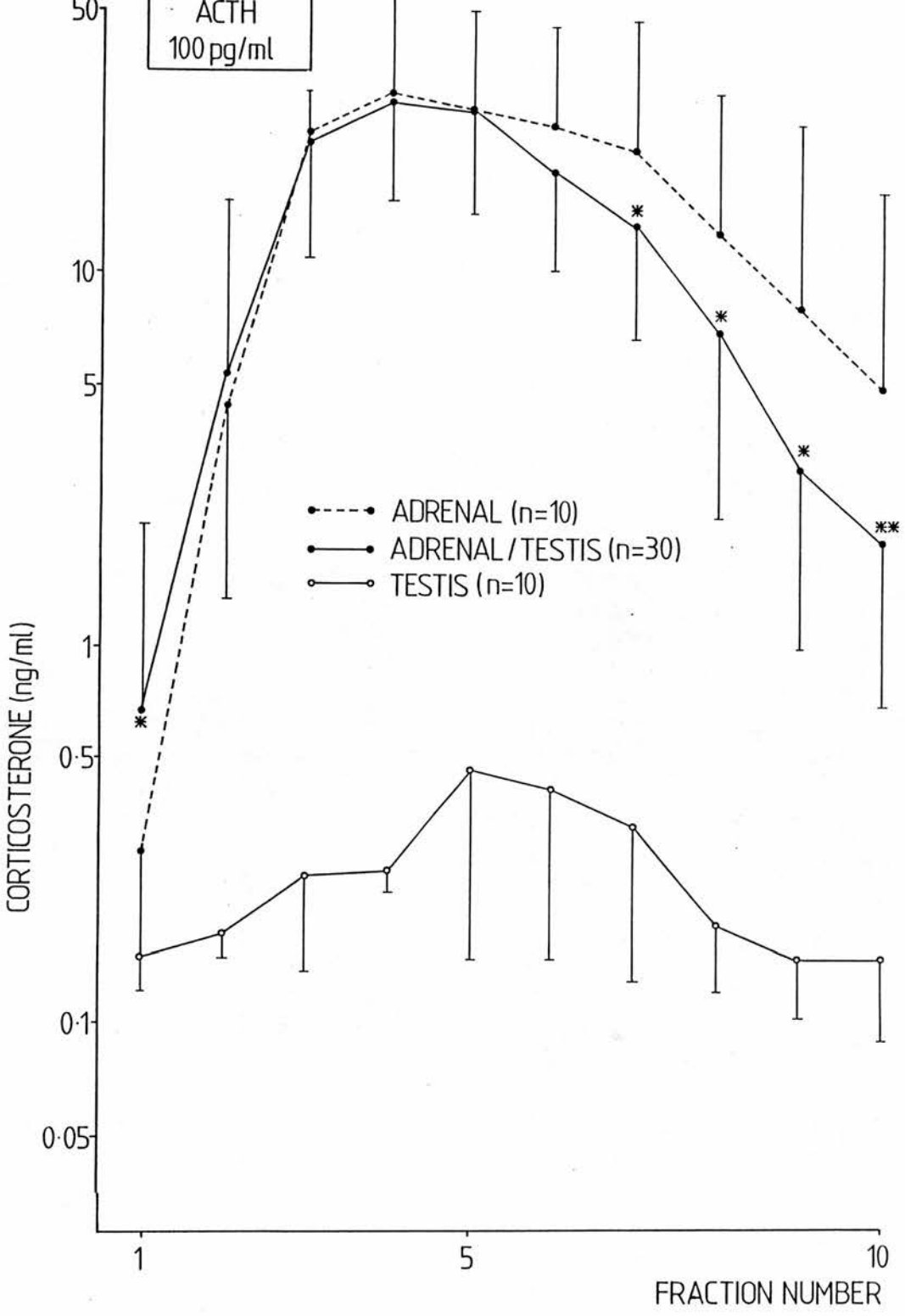


Figure 4.9: Pooled data from 5 separate experiments illustrating the corticosterone response to ACTH 100 pg/ml by isolated adrenal cells (●----●), testicular cells (○——○) and a mixed cell population (●——●). (* = $p < 0.05$, ** = $p < 0.005$ and *** = $p < 0.0005$).

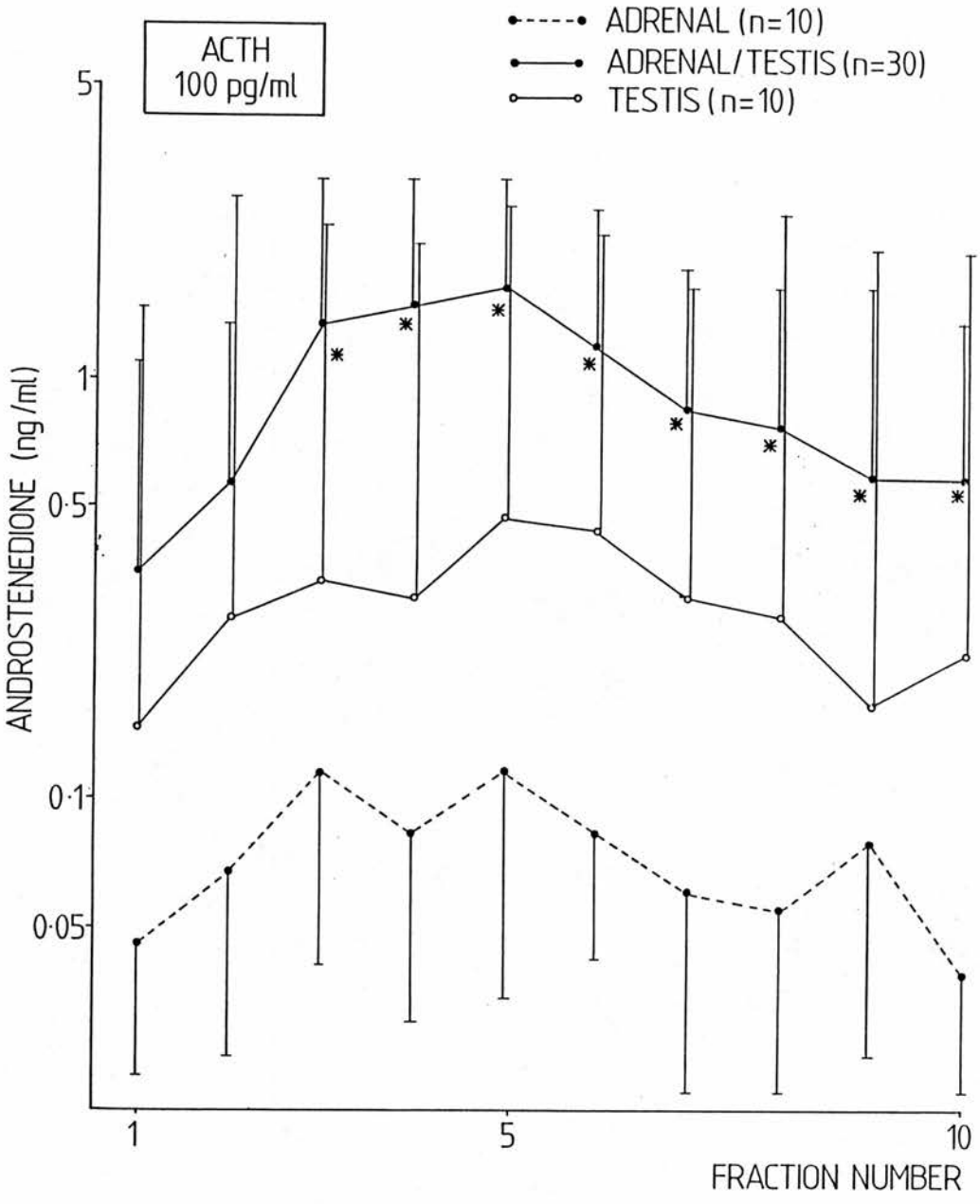


Figure 4.10: Pooled data from 5 separate experiments illustrating the androstenedione response to ACTH 100 pg/ml by isolated adrenal cells (●----●), testicular cells (○----○) and a mixed cell population (●----●). (* = $p < 0.05$, ** = $p < 0.005$ and *** = $p < 0.0005$).

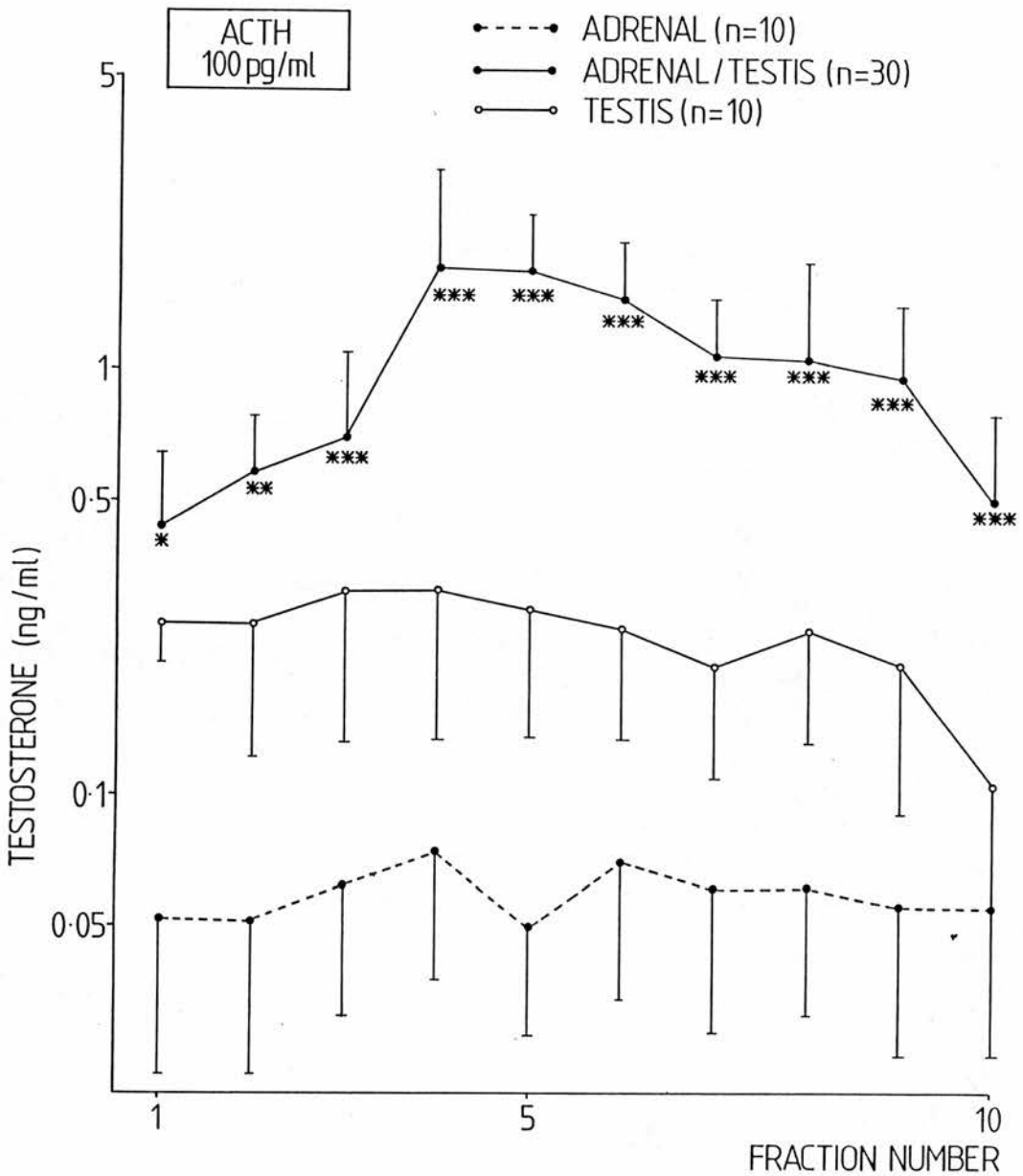


Figure 4.11: Pooled data from 5 separate experiments illustrating the testosterone response to ACTH 100 pg/ml by isolated adrenal cells (●----●), testicular cells (O——O) and a mixed cell population (●——●). (* = $p < 0.05$, ** = $p < 0.005$ and *** = $p < 0.0005$).

experiment). In contrast, secretion of these hormones by isolated testicular cells alone (n = 2 columns per experiment) was negligible. The stimulation of adrenal cells with ACTH increases the synthesis and secretion of adrenal steroid precursors that can directly stimulate testicular steroidogenesis. Furthermore, the adrenal steroids responsible for the stimulation of testosterone secretion must be those steroids secreted by the adrenal in the early pathway of adrenal steroidogenesis, occurring before or at the level of the synthesis of progesterone. The adrenal secretion of progesterone stimulates the testicular secretion of 17α-hydroxy-progesterone ➟ androstenedione ➟ testosterone.

The reduction of corticosterone in the eluate of the mixed cell population is an interesting observation that is perhaps a little difficult to explain, other than preferential utilisation of adrenal progesterone by juxtaposed testicular cells.

4.1.4: Response of isolated cells to progesterone and hCG

In order to determine whether the adrenal secretion of progesterone may act as a hormone precursor for testicular steroidogenesis, ACTH (100 pg/ml), hCG (50 ng/ml) and progesterone (10 ng/ml) was administered to either isolated testicular cells alone (n = 5 columns) or a mixture of isolated adrenal and testicular cells together (n = 5 columns). The importance of determining an *in vitro* response of testicular cells to hCG is to demonstrate that the preparation of isolated testicular cells was biologically viable with retention of LH-receptors following

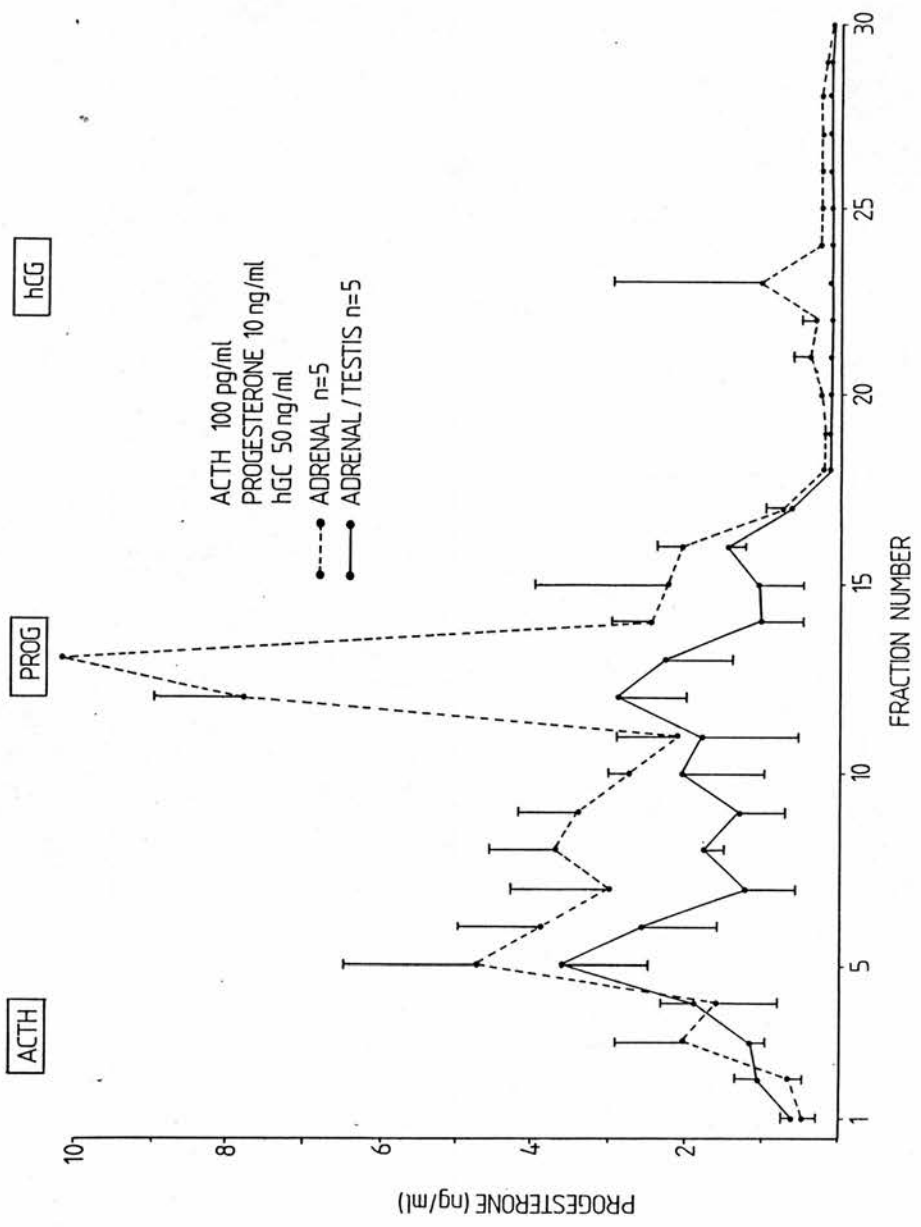


Figure 4.12: Mean ± SE progesterone response to ACTH 100 pg/ml, progesterone 10 ng/ml and hCG 50 ng/ml by isolated superfused adrenal cells (●----●) and mixed adrenal-testicular cell population (●——●).

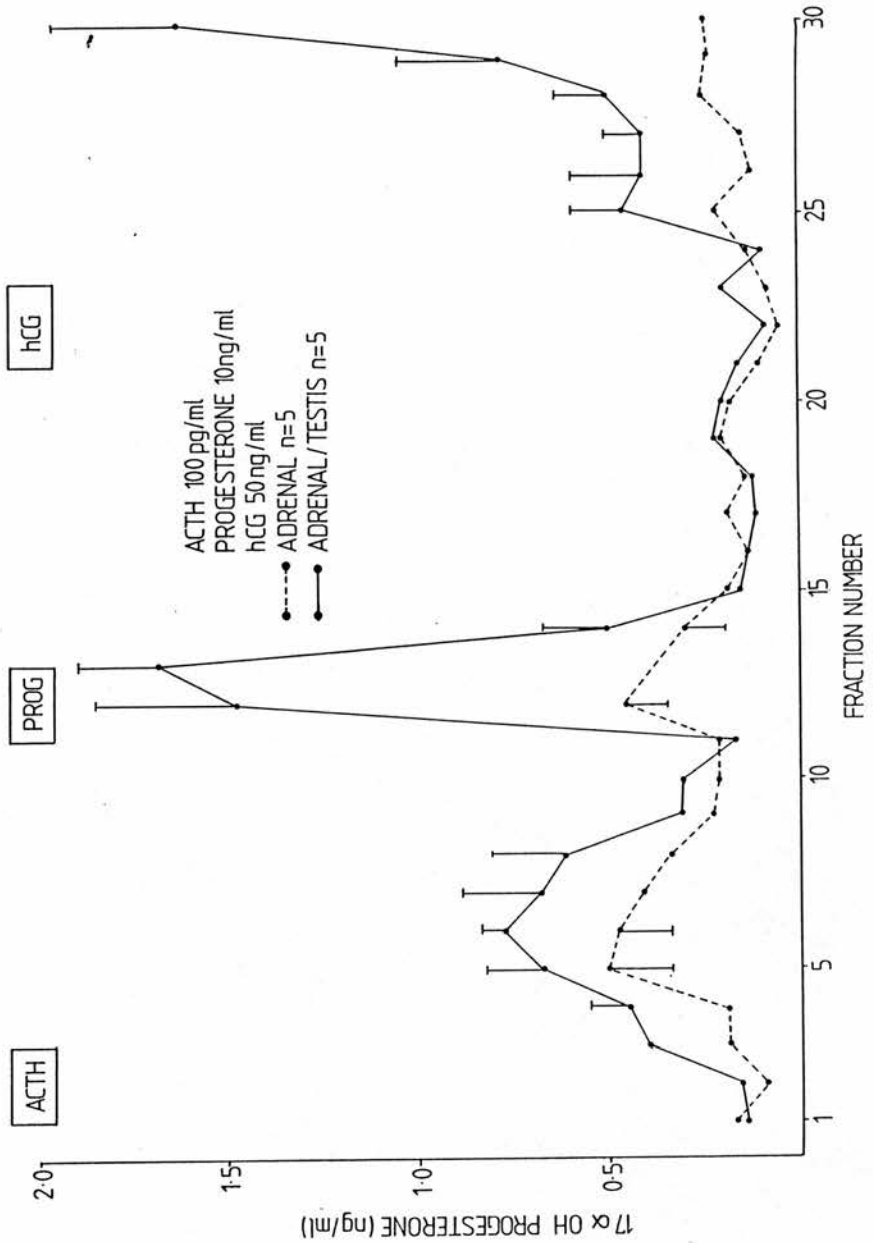


Figure 4.13: Mean ± SE 17α-hydroxyprogesterone response to ACTH 100 pg/ml, progesterone 10 ng/ml and hCG 50 ng/ml by isolated superfused adrenal cells (●----●) and mixed adrenal-testicular cell population (●——●).

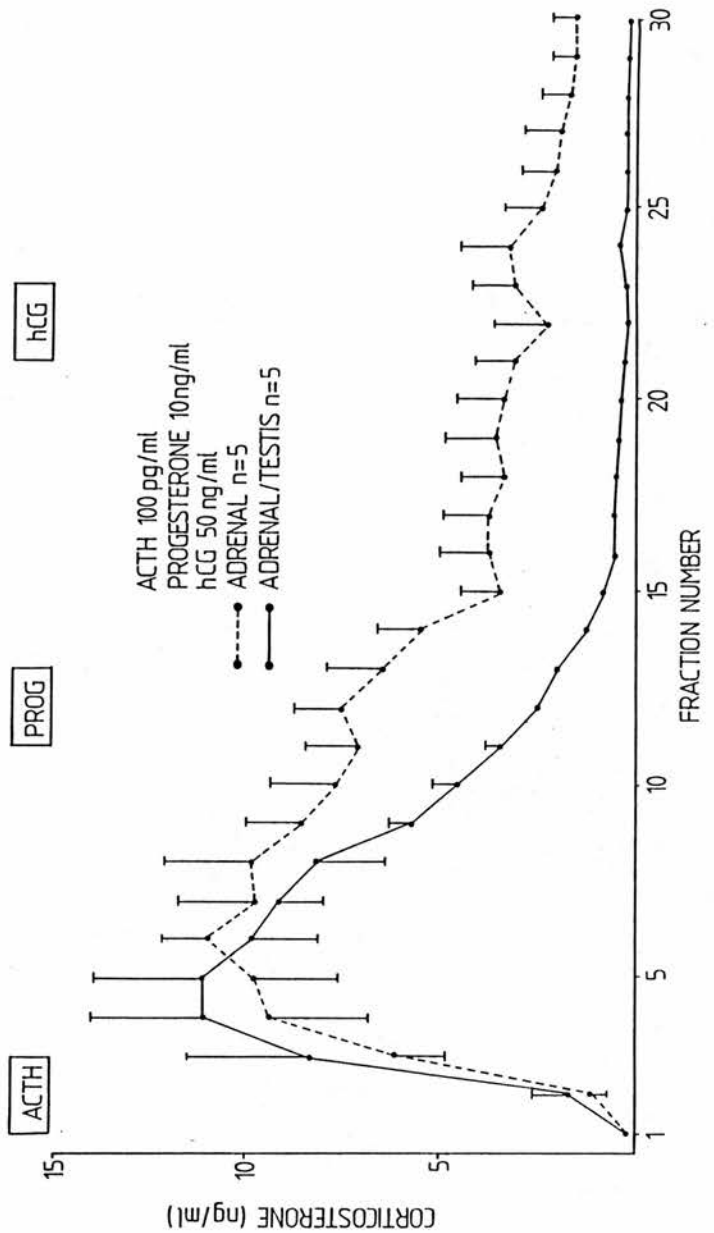


Figure 4.14: Mean ± SE corticosterone response to ACTH 100 pg/ml, progesterone 10 ng/ml and hCG 50 ng/ml by isolated superfused adrenal cells (●----●) and mixed adrenal-testicular cell population (●——●).

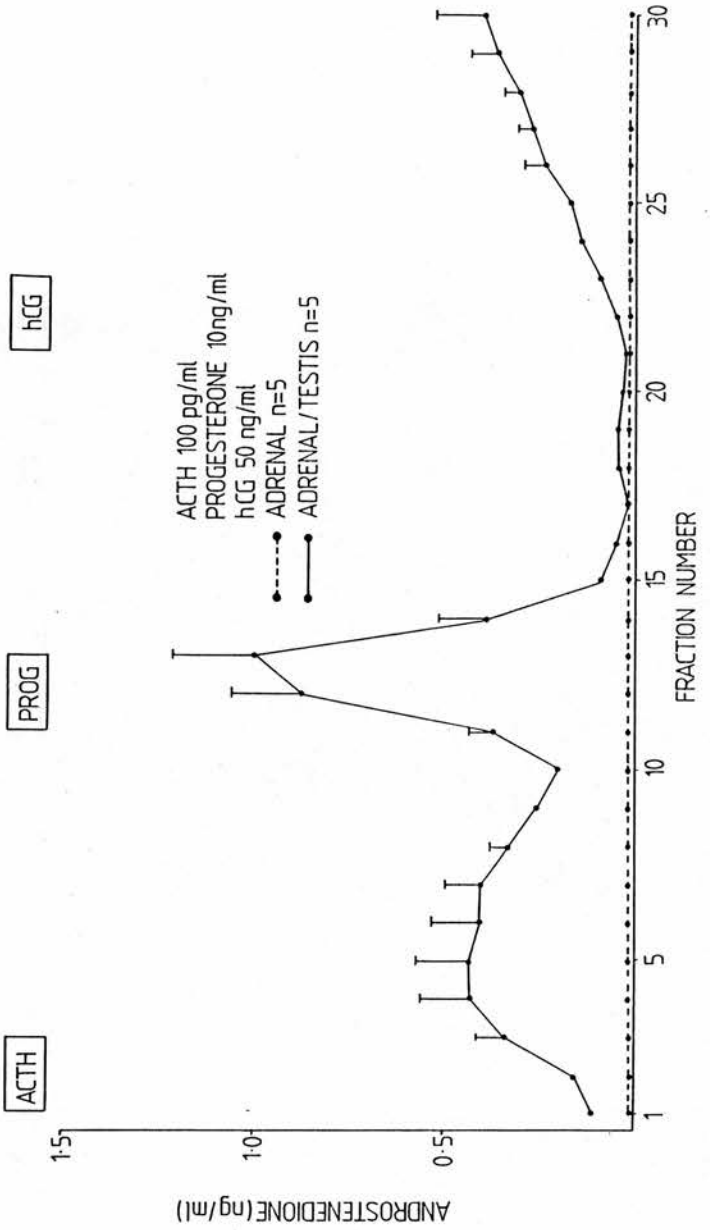


Figure 4.15: Mean ± SE androstenedione response to ACTH 100 pg/ml, progesterone 10 ng/ml and hCG 50 ng/ml by isolated superfused adrenal cells (●----●) and mixed adrenal-testicular cell population (●——●).

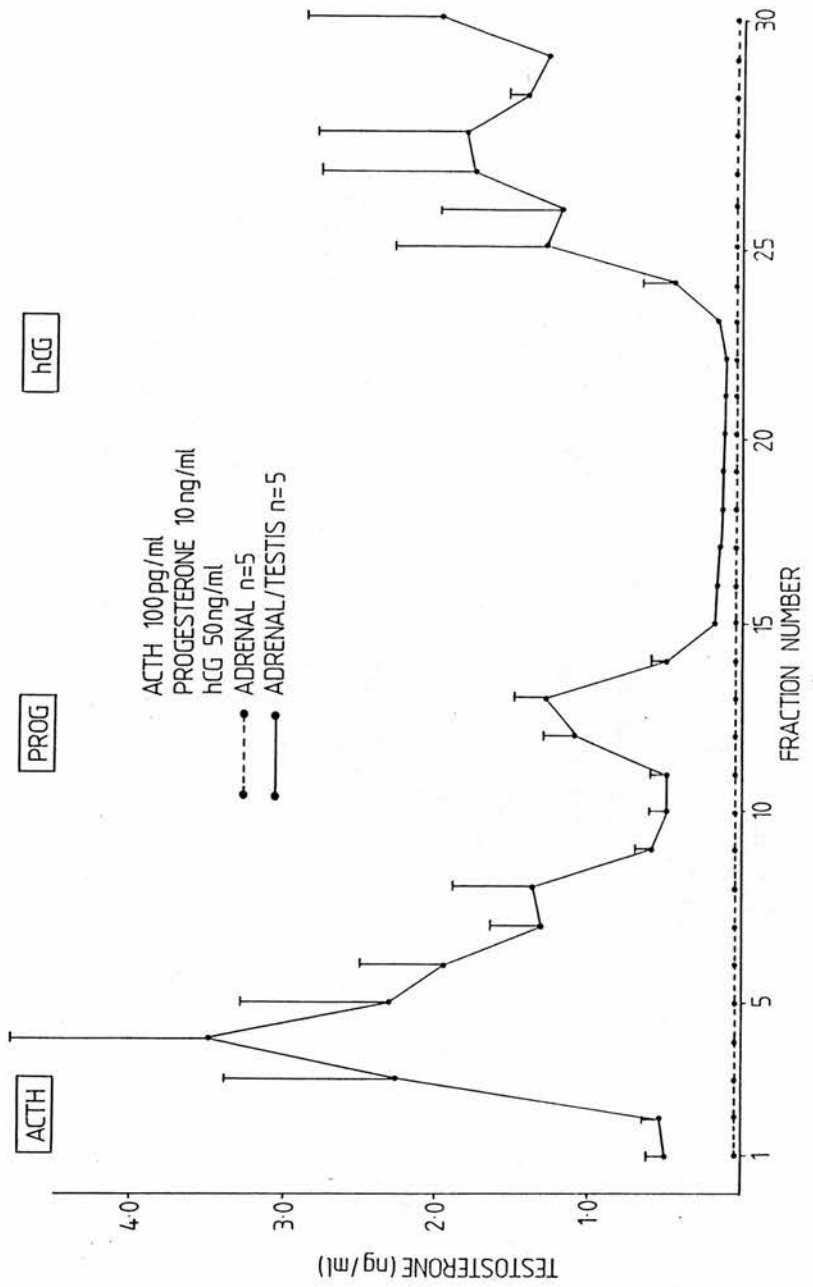


Figure 4.16: Mean ± SE testosterone response to ACTH 100 pg/ml, progesterone 10 ng/ml and hCG 50 ng/ml by isolated superfused adrenal cells (●----●) and mixed adrenal-testicular cell population (●——●).

collagenase digestion and that any response is not necessarily due to destruction of membrane integrity by enzyme digestion ('leaky cells'). The dose of hCG was chosen following work by Dr F C W Wu, M R C Reproductive Biology Unit, Chalmers Street, Edinburgh, in optimising the stimulation of isolated testicular cells in the *in vitro* superfusion system to the administration of both LH and hCG. Maximal stimulation of isolated testicular cells is usually achieved by this dose of hCG. The dose of progesterone was chosen to achieve similar concentrations of progesterone in the superfusion medium to that following the stimulation of isolated adrenal cells by ACTH 100 pg/ml. The results of a single experiment are illustrated in figures 4.11 to 4.16. Both ACTH and progesterone stimulated the secretion of 17α-hydroxyprogesterone, androstenedione and testosterone from the mixed cell population when compared with isolated adrenal cells alone. In comparison, concentrations of progesterone and corticosterone were reduced in the eluate of the mixed cell population when compared with isolated adrenal cells alone. This data supports the conversion of progesterone by isolated rat testicular cells *in vitro* ⟶ 17α-hydroxyprogesterone ⟶ androstenedione ⟶ testosterone.

However, the peak steroid response to the administration of progesterone is less than that achieved with ACTH-stimulated cells (mean $\pm$ SE testosterone concentration 1.3 $\pm$ 0.2 ng/ml for progesterone-stimulated cells compared to 3.52 $\pm$ 1.3 ng/ml for ACTH-stimulated cells), despite a higher concentration of progesterone achieved by the administration of progesterone (10

ng/ml) when compared with that achieved by ACTH-stimulated cells (3.66 $\pm$ 1.33 ng/ml). The implication of this observation is that if the adrenal secretion of progesterone is responsible for the stimulation of testicular steroidogenesis, other adrenal steroid precursors must be involved to achieve similar testosterone concentrations as that by ACTH-stimulated cells. The administration of pregnenelone (10 ng/ml), 17-hydroxypregnenelone (10 ng/ml) and 17α-hydroxyprogesterone to isolated testicular cells also stimulated the secretion of testosterone to concentrations approximately three times that of basal values (data not shown).

Whilst the administration of progesterone to the isolated mixed cell population stimulates testicular steroidogenesis, there does not appear to be stimulation of adrenal steroidogenesis. Further, the stimulation of isolated testicular cells in the mixture does not appear to stimulate adrenal steroid production. This implies that with the juxtaposition of isolated adrenal and testicular cells in the superfusion system the direction of adrenal-testicular cooperation is one-way from the adrenal ➟ testis. Alternatively, the amount of corticosterone secreted by adrenal cells in the eluate is approximately 10 times the amount of testosterone secreted in the eluate by testicular cells. Therefore, on a molar basis, the amount of steroid precursor required to produce a five-fold increase in testicular testosterone secretion may be insufficient to produce a noticeable change in adrenal corticosterone secretion.

4.2: Discussion

The isolated cell superfusion approach has demonstrated a direct interaction between steroid-secreting cells of the adrenal and the testis by the close juxtaposition of cells *in vitro*. One possible explanation for this response is the transfer of receptors from one cell type to the other during the preparation of isolated cells for *in vitro* use. Concentrated and purified LH-receptors have been succesfully transferred to isolated adrenal cells enabling them to respond to stimulation with LH by increased secretion of corticosterone *in vitro* (Dufau, 1978). However, the lack of response by testicular cells alone to the administration of ACTH would make this an unlikely explanation of these results.

Previous studies attempting to demonstrate the influence of the adrenal and its steroids over the testis *in vitro* have employed a variety of methods:

i) **Static incubations of isolated cells** have failed to demonstrate increased secretion of testosterone from a mixed cell population of isolated adrenal and Leydig cells in response to ACTH (Podesta, 1984). Similarly, the present study also failed to show increased testosterone secretion from a mixed population of adrenal and testicular cells in response to the administration of ACTH in static incubations (data not shown). This presumably reflects the disadvantages of static incubation when compared with the isolated cell superfusion. The possible reasons for these discrepancies have been previously discussed. However both Leydig and Sertoli cells are able to metabolise progesterone to testosterone under

these conditions (Hall, 1969; Tcholakian, 1978).

ii) **Superfusion of purified isolated Leydig cells** with pregnenelone results in the conversion ➡ progesterone ➡ 17α-hydroxyprogesterone ➡ androstenedione ➡ testosterone (Kühn-Velten, 1984), confirming the findings of the present study.

iii) **Electrofusion of isolated cells** produces a hybrid cell line that secretes corticosterone (mean corticosterone 160 pg/tube) in response to LH (10 iU/ml) and testosterone (mean testosterone 225 pg/tube) in response to ACTH (10 pM), whereas these isolated cells alone fail to secrete significant amounts of either corticosterone in response to LH, or significant amounts of testosterone in response to ACTH (Podesta, 1984).

iv) **Static incubation of whole glands** have been successful in demonstrating ACTH-mediated secretion of testosterone by the testis only when fetal adrenals are incubated in the presence of fetal testes and not when the two glands are separate (Warren, 1984b). Whole glands are also able to convert progesterone to testosterone in vitro (Steinberger, 1968).

There is therefore significant support for the idea that the adrenal secretion of steroid precursors may act as substrates for synthesis of testosterone by rat testicular Leydig cells in vitro. That the testis can metabolise progesterone is perhaps not surprising as both the adrenal and the testis share a common steroidogenic pathway.

The two major pathways of **testicular steroidogenesis** in the rat (Chubb, 1979b) are:

i) Pregnenelone ➡ 17-hydroxypregnenelone ➡ 17-hydroxy-progesterone ➡ androstenedione ➡ testosterone. This pathway accounts for up to 72% of the testosterone secreted by the rat testis with the rate-limiting step residing in the conversion of pregnenelone ➡ 17-hydroxypregnenelone.

ii) Pregnenelone ➡ progesterone ➡ 17-hydroxy-progesterone ➡ androstenedione ➡ testosterone. This pathway accounts for up to 57% of the testosterone secreted by the rat testis with the rate-limiting step in this pathway is the conversion of pregnenelone ➡ progesterone.

Thus pregnenelone can be metabolised by the two pathways at almost equal rates.

The two major pathways of **adrenal steroidogenesis** in the rat are:

i) Pregnenelone ➡ progesterone ➡ 11-deoxycorticosterone ➡ corticosterone ➡ (aldosterone).

ii) Pregnenelone ➡ 17-hydroxypregnenelone ➡ 17α-hydroxyprogesterone.

4.3: Conclusions

The significance of these in vitro findings is to provide evidence that:

i) only isolated testicular and not adrenal cells secrete androgens in any significant quantity.

ii) the adrenal secretion of steroid precursors and the administration of progesterone stimulates testicular

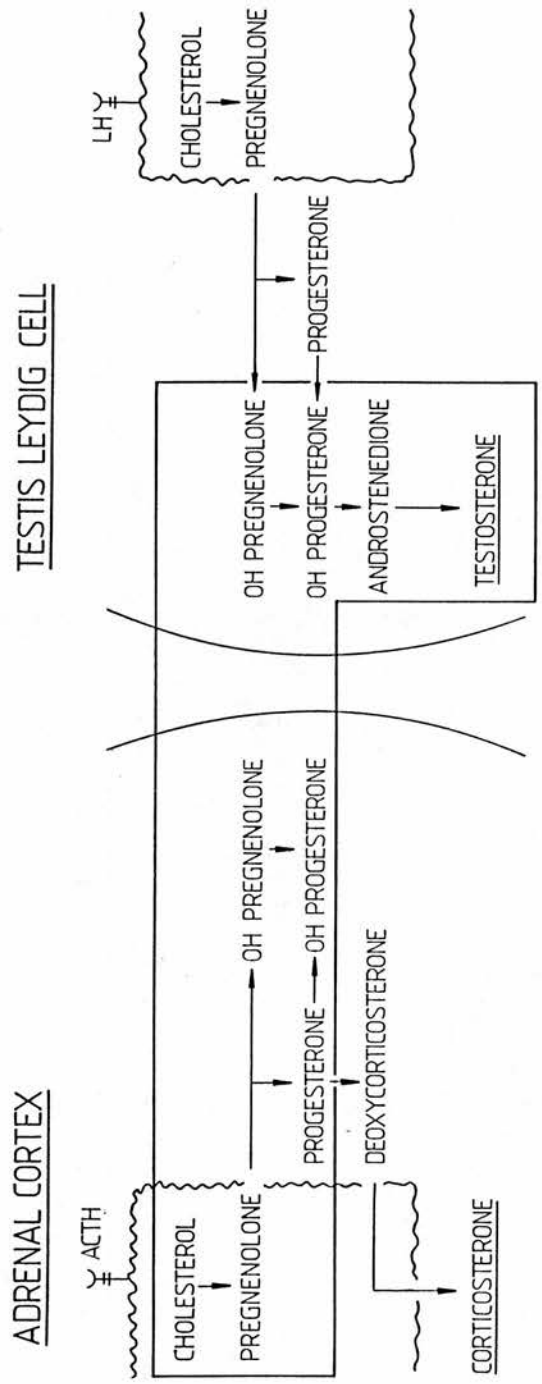


Figure 4.17: Adrenal ➡ gonad interaction <u>in vitro</u>

steroidogenesis (Fig 4.17).

iii) the concentrations of steroids achieved in the superfusion system are similar to the levels of those steroids *in vivo*.

Chapter 5

CONCLUSION

5.1: Conclusions

The adrenal secretion of progesterone and corticosterone act as two important external signals to the hypothalamic-pituitary-testicular axis (Fig 5.1):

i) **The adrenal synthesis of progesterone** and its subsequent secretion into the circulation provides a positive signal for testicular steroidogenesis and both Leydig and Sertoli cells are probably involved in the metabolism of progesterone to testosterone. However, the ability of progesterone to stimulate testicular steroidogenesis appears to be restricted to definite periods in testicular maturation - in fetal life, before and after puberty. The adrenal secretion of progesterone and its conversion to testosterone by the cytoplasm of the Leydig cell may provide an alternative pathway for testosterone biosynthesis during the acute phase of stress and mating.

ii) **The adrenal secretion of corticosterone** may provide an important negative signal to testicular steroidogenesis by inhibiting the anterior pituitary secretion of LH and may also have direct effects on LH-receptors of the Leydig cells, inhibiting testicular steroidogenesis directly. Whilst suppression of the hypothalamic-pituitary-testicular axis may be achieved with pharmacological levels of glucocorticoids, this may also be achieved with physiological levels (Vreeburg, 1984). The administration of ACTH to intact rats increases the adrenal secretion of both progesterone and corticosterone (achieving plasma corticosterone levels of approximately 300 ng/ml) and depresses

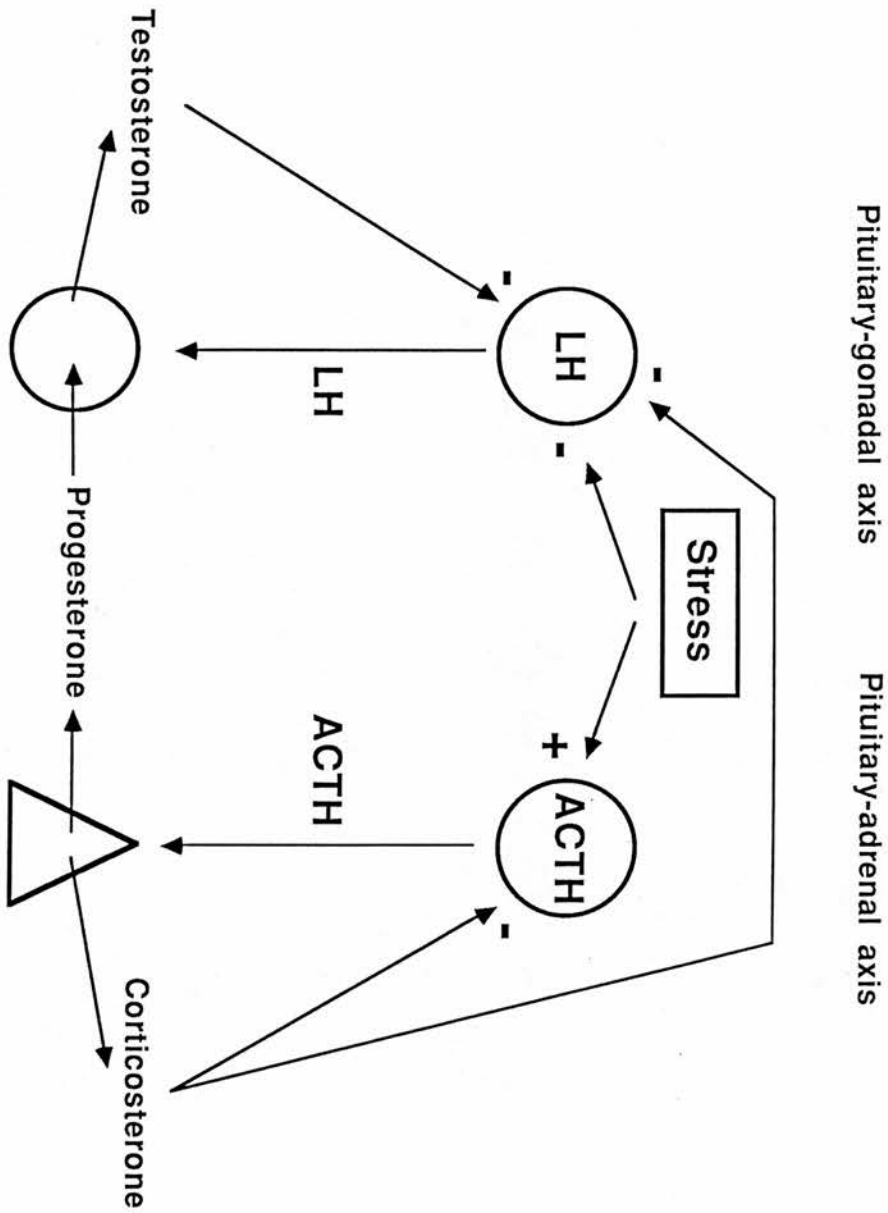


Figure 5.1: The adrenal gland provides two important external signals that may modify the internal signals of the hypothalamic-pituitary-testicular axis in the rat.

plasma testosterone levels.

5.2: Evidence for adrenal ➡ gonad interactions in the female rat.

So far this thesis has largely concentrated upon the influence of the adrenal upon gonadal function in the male rat. Yet there is evidence that the adrenal may affect the function of the female reproductive system in the rat. In the female, the adrenal gland appears to be necessary for the development of normal puberty, the development of vaginal opening, ovulation, lactation, pregnancy and the quality of the fetus (Thoman, 1970; for reviews see Ramaley, 1974; Ramaley, 1975; MacFarland, 1977).

Much of the information concerning the influence of the adrenal gland on female reproductive function has been derived from the effects of adrenalectomy on these parameters. It is quite clear that the hormonal response to adrenalectomy in female rats is very different from their male counterparts. In females, adrenalectomy enhances the castration-induced rise of the anterior pituitary secretion of LH, and inhibits the secretion of FSH (Zanisi, 1983). It would appear that a signal from the adrenal gland inhibits the anterior pituitary secretion of LH and loss of this signal facilitates the post-castration rise in serum LH concentrations.

The administration of ACTH to intact prepubertal females also delays the onset of puberty and is associated with increased plasma progesterone and depressed plasma LH levels, implying that

progesterone is the signal inhibiting ovarian function. Whilst adrenalectomy achieves a similar delay in the onset of puberty, plasma concentrations of corticosterone and progesterone were undetectable, suggesting that the mechanism of delayed puberty in these animals was different to that of their intact counterparts. Adrenalectomy may therefore delay the onset of puberty in female rats by non-specific means.

The fluctuations of LH, oestrogen and progesterone during the oestrus cycle is well characterised (for review see Kalra, 1977):

i) **Secretion of LH:** Low mean levels of LH are found in the blood at oestrus, increasing from oestrus through to dioestrus I due to increased pulse frequency and amplitude, then decreasing from dioestrus II through to the morning of pro-oestrus due to a decrease in pulse amplitude. During the afternoon of pro-oestrus the pre-ovulatory surge of LH is characterised by an increase in both amplitude and frequency of secretion similar to that seen only in the castrated female rat. Oestrogen is the primary ovarian signal responsible for the preovulatory release of LH and is mediated by a complex interaction acting at the level of the anterior pituitary gonadotroph and also possibly at the level of the hypothalamus. The effect of ovarian progesterone is probably mediated by its direct effect upon the pituitary gland although some evidence suggests that this may be centrally mediated. Under conditions of steady oestrogen concentrations progesterone stimulates LH secretion in low doses and inhibits in higher doses (McPherson, 1979).

ii) **Sex steroids:** The oestrus phase of the cycle is a relatively quiescent period for ovarian steroid production but oestrogen and progesterone secretion increase from dioestrus I until the noon of dioestrus II, when ovarian progesterone secretion wanes, and oestrogen secretion gradually increases culminating in a rapid peak around the time of the pre-ovulatory surge in LH secretion (pro-oestrus). Ovarian progesterone acts to restrain oestrogen secretion as the ovarian follicles grow from dioestrus I through to dioestrus II. Blood levels of progesterone must remain low during the later phase of dioestrus to allow the positive feedback of oestrogen effect on LH secretion. Similarly the adrenal secretion of progesterone and other precursor steroids may be important in modulating LH release. Both ACTH and deoxy-corticosterone have been shown to stimulate the surge of gonadotrophins underlying ovulation (Kraulis, 1978; McPherson, 1979).

The fundamental difference in the effect of these steroids on the anterior pituitary secretion of gonadotrophin in the sexes probably depends upon the sexual differentiation of the hypothalamus (Brown-Grant, 1974; Aiyer, 1976; Fink, 1977; for review see Kalra, 1983).

5.3: Evidence for adrenal ⟶ gonad interactions in the male of other animal species.

i) **In the rabbit,** the acute administration of ACTH to intact animals results in a rapid rise of plasma corticosteroid levels

and a biphasic response of plasma testosterone levels - an initial rise is followed by a fall over a period when elevation of plasma corticosteroids is sustained (Pitzel, 1984). This response is not seen in castrated rabbits.

ii) **In the pig,** the acute administration of ACTH to intact boars results in a rapid rise in plasma corticosteroid, progesterone and testosterone concentrations that is not seen in their adrenalectomised counterparts or in castrated boars (barrows) (Liptrap, 1975; Hahmeier, 1980; Juniewicz, 1981; Juniewicz, 1984). Furthermore, this response is not mediated via LH secretion (Juniewicz, 1981). In contrast, the chronic administration of ACTH suppresses plasma testosterone levels indirectly by inhibiting anterior pituitary gonadotrophs (Liptrap, 1968; Liptrap, 1975). Plasma concentrations of corticosteroids and testosterone rise sharply in boars exposed to another aggressive boar or during copulation with an oestrous sow. The administration of metyrapone reduces this response without interfering directly with testicular steroidogenesis (Liptrap, 1978). However, the administration of lysine-vasopressin failed to mimic the results achieved by the administration of ACTH despite inducing a rise in plasma corticosteroid concentrations. Nevertheless, it is tempting to speculate that stress-induced elevation of plasma testosterone concentrations is important in the sexual and aggressive behaviour of these animals.

These studies indicate a strong relationship between the adrenal secretion of corticosteroids and testicular steroidogenesis

that is independent of anterior pituitary LH secretion. However, there is conflicting data on the results of the acute administration of corticosteroids to these animals. The acute administration of cortisol, in a dose achieving physiological plasma levels, results in a rapid rise in plasma testosterone levels (Liptrap, 1975). However, this observation has not been confirmed by other investigators (Juniewicz, 1981). In contrast, the administration of pharmacological doses of glucocorticoid suppresses testicular steroidogenesis by inhibition of LH-secretion (Liptrap, 1968).

iii) **In dogs** it has been demonstrated that perfusion of the testis in situ with the animal's own adrenal venous blood resulted in a greater secretion of dehydroepiandrosterone and testosterone than when the testis was perfused with the animal's own peripheral arterial blood (Wassermann, 1969). Since the canine adrenal does not secrete significant amounts of either dehydroepiandrosterone or testosterone it is likely that an adrenal precursor such as progesterone stimulates testicular steroidogenesis.

5.4: Evidence for adrenal ➡ gonad interactions in the human

Recent studies have attempted to determine whether the adrenal secretion of corticosteroids has any influence over gonadal function in human health and disease.

i) **In men** acute stress increases the concentrations of ACTH, cortisol, androstenedione and dehyroepiandrosterone but depresses the concentration of testosterone in the blood. The fall in plasma

testosterone is probably mediated by the acute rise in plasma cortisol concentrations since the acute administration of hydrocortisone achieves a similar decline (Cumming, 1983).

The chronic administration of ACTH to normal males results in increased secretion of cortisol, androstenedione and a profound suppression of testosterone secretion without influencing plasma LH levels (Rivarola, 1966; Beitins, 1973; Smals 1974; Irvine, 1974). Similarly chronic stress, illness, surgery under general anaesthesia and vigorous exercise are also associated with marked decreases in plasma testosterone concentrations (Wang, 1978a; Wang 1978b; for review see Cumming, 1983). It would appear that the adrenal secretion of corticosteroids plays a major inhibitory role over the hypothalamic-pituitary-testicular axis in normal man.

However, the administration of dehydroepiandrosterone sulphate to boys with familial cytomegalic adrenocortical hypoplasia significantly increases plasma levels of dehydroepiandrosterone, its sulphate, androstenedione and testosterone concentrations (Cohen, 1982). Whether these plasma levels reflect direct stimulation of testicular steroidogenesis by dehydroepiandrosterone or its peripheral conversion is unknown. The testis is the major contributor to plasma testosterone concentrations (for review see Brooks, 1984). The respective daily secretion rates of androgen by the human adrenal and testis are 24 and 0.7 μmol for dehydroepi-androsterone, 7 and 0.7 μmol for androstenedione and 0.04 and 24 μmol for testosterone. It is possible that only the suppressed testis is capable of responding to the administration of steroid

precursors.

ii) **In women** a great deal of attention has been focused on the possibility that the adrenal gland may profoundly affect the ovary in the polycystic ovarian syndrome (for review see Yen, 1980 and Brooks, 1984). The clinical mainifestations of this disorder are the continuation of postmenarchal menstrual irregularity and progressive development of hirsutism, obesity, infertility and the development of polycystic ovaries. In the blood, the characteristic features are raised plasma LH and depressed plasma FSH levels in association with raised plasma androgen concentrations (dehydroepiandrosterone, androstenedione and testosterone) and raised levels of plasma oestrogen (in particular an increased ratio of oestrone : oestradiol).

Although it is well established that plasma androgen levels are raised in this condition, the source of these raised levels has been the centre of a great deal of controversy. Venous catheterisation studies have demonstrated an adrenal source for dehydroepiandrosterone and an ovarian source for androstenedione and testosterone. This is confirmed by the ready suppressibility of plasma dehydroepiandrosterone but not plasma androstenedione and testosterone levels by the administration of pharmacological doses of dexamethasone (Lachelin, 1982). Thus polycystic ovaries are probably the source of excessive amounts of androstenedione which is converted by peripheral conversion to both oestrone and testosterone. The former stimulates LH and suppresses FSH secretion leading to a vicious cycle of chronic anovulation, the

development of polycystic ovaries and continuing increased androgen secretion. The latter leads to hirsutism and obesity (Yen, 1980).

However, adrenal scintigraphy has shown that patients with polycystic ovaries have increased adrenocortical function (Gross, 1986). Furthermore, plasma pregnenelone, progesterone, 17-hydroxyprogesterone and dehydroepiandrosterone concentrations are significantly increased in response to ACTH in patients with polycystic ovaries (Lachelin, 1979) as are the levels of 11-deoxycortisol, androstenedione and testosterone in response to metyrapone (for review see McKenna, 1985; Loughlin, 1986).

The conclusion of these studies is that there appear to be two major abnormalities in the polycystic ovarian syndrome:

i) abnormal adrenal function with the increased secretion of steroid precursors.

ii) abnormal polycystic ovaries that secrete abormal amounts of androstenedione.

A unifying hypothesis to explain this syndrome is that an abnormal adrenarche with increased secretion of adrenal androgens is essential in the initiation of of the abnormal gonadotrophin response and the subsequent development of polycystic ovaries. In the early stages of this condition suppression of the hypothalamic-pituitary-adrenal axis with dexamethasone will restore the abnormal hormonal profiles to normal and initiate normal menstrual function. However, as androgen production from the ovary increases in response to the abnormal gonadotrophin pattern, suppressibility of this condition with dexamethasone becomes reduced (Loughlin, 1986).

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